

Studies on Cartilage Proteoglycans in Aging and Degenerative Joint Disease

Sven Inerot



Lund 1983



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Studies on Cartilage Proteoglycans
in
Aging and Degenerative Joint Disease

Sven Inerot

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To Annica, Niclas and Eva

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LIST OF PAPERS

This thesis is based on the following papers referred to in the text by their Roman numerals.

- I. Articular-Cartilage Proteoglycans in Aging and Osteoarthritis.
Sven Inerot, Dick Heinegård, Lars Audell, and Sten-Erik Olsson
Biochem. J. (1978) 169, 143-156
- II. Variation in the Composition of Bovine Hip Articular Cartilage with distance from the Articular Surface.
Ahnders Franzén, Sven Inerot, Sven-Olof Hejderup and Dick Heinegård
Biochem. J. (1981) 195, 535-543
- III. Bovine Tracheal Cartilage Proteoglycans. Variation in Structure and Composition with age.
Sven Inerot and Dick Heinegård
Collagen Rel. Res. (1983) 3. 245-262
- IV. Structure and Composition of Articular Cartilage Proteoglycans in Experimental Osteoarthritis.
Sven Inerot, Dick Heinegård, Sten-Erik Olsson, Hans Telhag and Lars Audell
J. Orthop. Res. (1983) Submitted for publication.
- V. The Normal Variation in Structure and Composition of Canine Hip Articular Cartilage Proteoglycans.
Sven Inerot and Dick Heinegård
In manuscript.

VI. Quantitation of Cartilage Proteoglycan Structures Liberated to the Synovial Fluid during Developing Degenerative Joint Disease.

Dick Heinegård, Sven Inerot, Jörgen Wieslander and Gert Lindblad

J. Orthop. Res. Submitted for publication.

ABBREVIATIONS

CS	- chondroitin sulfate
CS-4	- chondroitin 4-sulfate
CS-6	- chondroitin 6-sulfate
ELISA	- enzyme linked immunosorbent assay
HA	- hyaluronic acid
KS	- keratan sulfate

BACKGROUND

Cartilage

Cartilage is a specialized form of connective tissue, having the main function of providing flexibility to the skeletal system. Cartilage provides elasticity and resistance to compressive forces and ensures smooth articulation at the joint surfaces. Most cartilages are of similar molecular composition, containing water and two major types of macromolecules, collagen and proteoglycans. Collagen forms a three dimensional fibrillar network, that determines the maximal volume of the tissue, i.e. hinders its further expansion by tensile restraint. The proteoglycans, which are entrapped in the collagen network, are the molecules in the body having the highest density of negatively charged groups. These molecules provide osmotic pressure and thereby forces striving towards expansion of the tissue against the tension of the collagen fibers. As a consequence cartilage is already in the normal state well suited to withstand compression (for refs. see 144). Another consequence of the composition of cartilage is restricted waterflow in the tissue. This is also of great importance for the resistance to deformation. Unique to articular cartilage is the lubrication of the articulating surfaces that depends on the extrusion and imbibition of water out of and into the cartilage (for refs. see 92).

A better understanding of cartilage function, however, requires more detailed knowledge of the organization of its macromolecules. Furthermore, the cells, named chondrocytes, although few and sparsely distributed in the tissue, have a fundamental role in organizing the tissue and probably also in regulating synthetic and degradative processes which are of significance in both health and disease.

Cartilage may be divided into three different types, elastic, fibrous and hyaline. The latter is typically found at the articulating surfaces of joints, in tracheal rings, in the larynx, in nasal septum, in the ventral portions of the ribs and in the young individual also in the epiphyses.

Hyaline cartilage contains water (60-80% of wet weight), proteoglycans (20-40% of dry weight) and collagen (40-70% of dry weight). The remainder is composed of other proteins, lipids, nutrients and inorganic material. Cellular material represents less than 10%, showing that the matrix forms the major part of the tissue.

Proteoglycans and collagen are thus the major constituents of cartilage. These molecules change during life, possibly to meet the altered functional requirements. Since they contribute essential properties to the tissue they are likely to be involved in disease processes leading to impaired cartilage function, for instance osteoarthritis. Increased knowledge of the structure of these macromolecules provides the basis for a better understanding of their function and of the nature of disease processes resulting in cartilage destruction. A brief review of these macromolecules is therefore presented below.

Proteoglycans

The cartilage proteoglycans are large, extended molecules having molecular weights of 1 to 4×10^6 . In general they consist of a central protein core with large numbers of side chains of chondroitin sulfate (CS), keratan sulfate (KS) and two types of oligosaccharides that are N-linked and O-linked, respectively (for refs. see 39).

The protein core can be divided into three regions, based on type of side chains and amino acid composition (see Fig. 1). The hyaluronic acid (HA) binding region, at one end, is substituted with N-linked oligosaccharides. At the other end of the proteo-

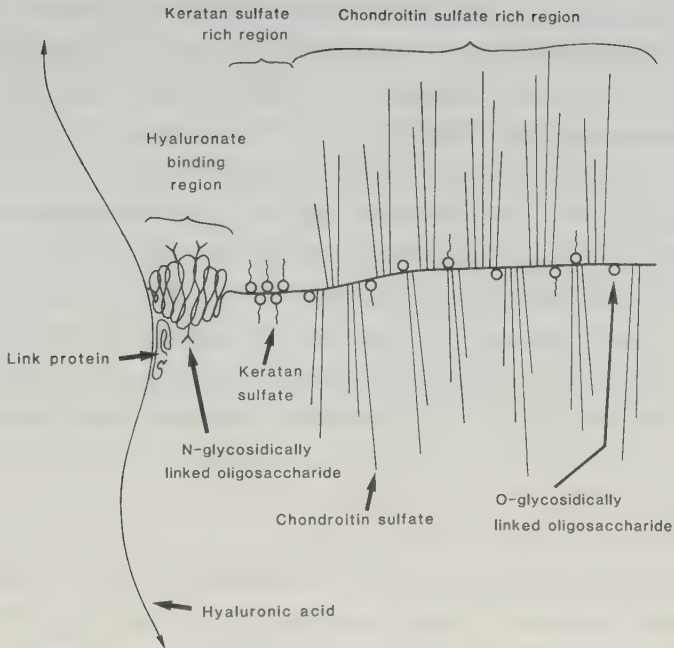


Figure 1. Tentative model of a cartilage proteoglycan monomer bound to hyaluronic acid. The binding is stabilized by link proteins.

glycan, one finds the CS rich region containing on average 100 chains of CS, some KS chains and probably O-linked oligosaccharides, structurally related to the linkage region of KS to protein. The third region, the KS- rich region located between the other two regions, contains KS chains and O-linked oligosaccharides (for refs. see 43).

CS is built from a repeat disaccharide of D-glucuronic acid and N-acetylgalactosamine, the latter containing a sulfate substituent at either the 4- or the 6-position. The chain containing some 40-50 such disaccharides is bound by an O-glycosidic linkage to the protein core via a specific linkage sequence of monosaccharides (for refs. see 43).

KS is built from a repeat disaccharide of galactose and N-acetylglucosamine substituted at the 6-position with sulfate. This glycosaminoglycan is smaller, sometimes containing as few as 4 or 5 disaccharide units depending on its origin. Skeletal

KS is linked via a specific linkage region to the core protein, with an N-acetylgalactosamine in the reducing terminal which forms an O-glycosidic linkage to serine or threonine (121, 17).

The proteoglycans containing the HA-binding region have the ability to specifically interact with hyaluronic acid. This interaction is stabilized by the additional binding of link proteins to the proteoglycan and to HA (38, 34, 28). Several proteoglycan monomers bind to one molecule of HA to form large aggregates with molecular weights in the order of 100×10^6 .

The cartilage proteoglycans are highly polydisperse. The molecules show a wide distribution of size (137) and a variability in sedimentation properties (108). It appears that the larger the molecule, the higher the CS content (108, 137, 41). Most of the polydispersity is probably caused by a variation in size of the CS-rich region while the KS-rich region and HA binding region appear to be constant (41, 145).

The composition of cartilage proteoglycans also varies with their source. Those from articular cartilage do on the average contain more KS and protein compared to those from tracheal and nasal cartilage (108).

In addition to the polydispersity with regard to size, the proteoglycans show molecular heterogeneity. Thus agarose-polyacrylamide gel electrophoresis demonstrate the presence of at least two components in proteoglycan preparations from several types of cartilage (63, 10, 130). They correspond to two distinct high molecular weight populations of aggregating proteoglycans which have been isolated from nasal cartilage (44). The larger and on agarose polyacrylamide gel electrophoresis more slowly migrating proteoglycan contains a predominant CS-rich region and less of KS, while the smaller, more slowly migrating proteoglycan contains a more prominent KS-rich region as well as a CS-rich region.

A third population of high molecular weight cartilage proteoglycans can not interact with HA and is therefore referred to as the non-aggregating proteoglycans (42). Another proteoglycan (apparent molecular weight 76 000) which lacks the ability to aggregate with HA can be isolated from cartilage. This proteoglycan contains one to two large CS chains and has a high content of protein (45). It has a monodisperse protein core (apparent molecular weight 45 000) which is very similar to that found in the low molecular weight proteoglycans from sclera, tendon, aorta and cornea. Such small proteoglycans may represent a class of ubiquitous molecules having a similar core protein (43).

Collagen

The basic unit of the collagen molecule is tropocollagen, which is a long rodlike molecule consisting of three polypeptide chains intertwined in a low pitch helix. Every third amino acid is a glycine. Other abundant amino acids are lysine, proline and hydroxy proline, the latter being unique and important for the conformational stability of the collagen. The polypeptide chains, called α -chains are found in different variants in the collagen of different tissues. Hyaline cartilage, for instance, contains three identical α_1 -chains and this collagen is referred to as type II. Collagen fibrils or fibers are formed in the matrix by side to side alignment of many tropocollagen molecules in a quarter staggered fashion. The collagen fibers are stabilized by crosslinks between the polypeptides. Such crosslinks become more abundant and chemically more resistant with increasing age. Additional minor collagen types have recently been reported to be present in cartilage. For further information on collagen biochemistry the reader is referred to recent reviews by von der Mark et al. (78), Piez (105) and Kivirikko (55).

Cartilage metabolism

Most constituents of the cartilage matrix are synthesized by the chondrocytes. Among the major components, the synthesis of collagen in adult cartilage is very low as is its degradation. The low level of collagen synthesis, which exists, is probably mainly due to repair processes (106).

The proteoglycans, have a higher rate of synthesis in adult tissue. Maroudas (80) has estimated the turnover times of adult articular cartilage proteoglycans as 2-3 years in humans and about 1 year in the rabbit and dog. It has been shown by in vitro and in vivo experiments that there appears to be two metabolic pools of cartilage proteoglycans in the tissue with turnover times of 3 days and from 60 up to several hundred days, respectively (35, 60, 80). Furthermore, Triphaus et al. (138) showed that human xiphoid cartilage contains two metabolically distinct populations of proteoglycans which differ with respect to their relative content of CS and KS. These pools may correlate to the two subpopulations of aggregating proteoglycans discussed above.

Available evidence can be taken to indicate that proteoglycans are eliminated from the tissue by progressive fragmentation by the action of proteolytic enzymes (7). Thus McDevitt et al. (68) obtained evidence that the proteoglycans in the tissue gradually become smaller, possibly by the action of proteolytic enzymes. The proteinases required can be provided by the chondrocytes that contain enzymes capable of proteolytic degradation of proteoglycans, i.e. neutral proteinases as well as cathepsins (for refs. see 7, 21).

Normal variation in articular cartilage composition with localization, depth and between individuals.

From earlier studies it is known that the composition of the articular cartilage varies with topographical localisation within the joint. Weightbearing cartilage on the superior region of the femoral head is thicker, has a higher glycosaminoglycan content and lower water and collagen contents than non-weight bearing cartilage. CS and KS contents showed a variation with localisation similar to that of the glycosaminoglycans (11, 141, 136). There are no or minor differences between the proteoglycans from different sites and different joints with respect to chemical composition, hydrodynamic size, ability to aggregate with HA, and CS chain length (13, 112).

The composition of articular cartilage also varies with distance from the articular surface. The water content decreases from the articular surface towards the osteochondral junction (82, 83, 142). Also the collagen content decreases somewhat from the surface towards the bone when expressed on a dry weight basis (142). Furthermore, the content of proteoglycans increases with depth from the articular surface to reach its maximum values in the midportions of the cartilage and it then decreases towards the osteochondral junction (131, 82, 83, 59). The structure of the proteoglycans also varies with increasing depth in the cartilage. They have somewhat higher KS and protein contents with increasing depth from the articular surface, while the CS content is lower (142, 10). These changes in composition are consistent with the observed decreasing size of the proteoglycans (10).

From previous studies (3, 82, 11) it is known that there are large variations between individuals in contents of total glycosaminoglycans, HA, CS and KS in full thickness articular cartilage. By contrast the water and collagen contents seem to be more constant (3, 88, 15, 82, 83). There are, however, no available studies of variation in proteoglycan composition and structure in

articular cartilage from the same localisation in the right and left side of the body in the same individual. Therefore it is not presently understood whether the variability between individuals is an effect of different load characteristics on different sites or joints, or just represents normal biological variability.

Cartilage in aging

Mechanical Properties. Marked changes in biomechanical properties of cartilage with advancing age have been described. Kempson (51, 52) and Roth and Mow (109) found that the tensile strength of articular cartilage decreases with increasing age, possibly due to an increased cross-linking of the collagen fibers (119). Also the elastic properties of cartilage are altered in the older individual, as shown by Armstrong et al. (4), who found that the deformation of articular cartilage under load increases with age. Kratovits (56) in a study of human femoral heads observed a definite decrease in cartilage stiffness with increasing age. The resistance of articular cartilage to compression is determined by the proteoglycan content. Alterations in proteoglycan structure may also be partly responsible for the changes in cartilage compressibility.

Composition. In normal articular cartilage there are only minor compositional changes with increasing age and the content of glycosaminoglycans per dry weight seems to be rather constant (3, 88, 15, 82). The water contents, however, have been found to decrease with age for full thickness articular cartilage (140, 83).

The relative proportions of KS and HA in hyaline cartilage increase while the proportion of CS decreases, with increasing age (50, 84, 124, 11, 140, 25, 95, 10). Furthermore, the ratio of

CS-6 to CS-4 increases with increasing age (84, 94, 111). Taken together these data indicate that the structure of the proteoglycans also changes with age.

Structure of cartilage proteoglycans. In most studies the cartilage proteoglycans have been extracted with concentrated chaotropic salt solutions and then purified by density gradient centrifugation in CsCl-gradients. A consistent finding is decreased extraction yields of proteoglycans with increasing age (124, 100, 111, 112, 116). In the most elderly the extracted proteoglycans represent only about 50-60% of the total proteoglycans while in the youngest 80-90% are extracted.

Furthermore, the structure of the isolated proteoglycans changes markedly with age and maturation. With increasing age there is a decreasing hydrodynamic size (9, 48, 133, 111, 112). The capacity of the proteoglycans to interact with HA and thereby form aggregates remains rather constant with age in articular cartilage (9, 111, 112). The composition of the proteoglycans also changes with age. Increasing glucosamine and protein contents and decreasing galactosamine contents reflect changes with regard to KS and CS respectively (124, 8, 9, 111, 112). The size of the CS side chains decreases slightly with increasing age (6, 133, 32, 136, 112). The KS chains on the other hand become somewhat larger with age and probably also more numerous, whereas the content of O-linked oligosaccharides, which are structurally related to the KS linkage region, decreases (133, 32, 112, 136, 116).

The observed changes in proteoglycan composition and structure can be explained if it is assumed that the HA binding region and the KS-rich region remain constant in size while the CS-rich region becomes smaller with increasing age (9, 48, 111). In support Sweet et al. (133) found that the size of the protein core decreases during maturation.

At least in some cartilages, the altered proteoglycan structure is partly due to a change in the relative proportion between the two subpopulations of aggregating proteoglycans described above. Triphaus et al. (138) using $[^{35}\text{S}]$ -labeling in vitro observed that human xiphoid cartilage with increasing age synthesized progressively more of a KS-rich population of small hydrodynamic size, while synthesis of a larger proteoglycan rich in CS decreased. Stanescu (129) in support, using agarose-polyacrylamide gel electrophoresis showed that articular cartilage of young individuals contained a somewhat larger proportion of proteoglycans of low mobility while cartilage from older individuals had a higher proportion of proteoglycans with higher electrophoretic mobility.

Metabolism of cartilage proteoglycans. As discussed above Triphaus et al. (138) showed an increased synthesis in vitro of smaller KS rich proteoglycans with increasing age. Others (100, 101) using explant cultures of cartilage similarly showed increased synthesis of smaller proteoglycans having a lower proportion of CS with increasing age of the animal. These proteoglycans retained the ability to interact with HA. The quantitative aspects of proteoglycan synthesis have been studied by Maroudas (79) who showed that the in vivo incorporation of $[^{35}\text{S}]$ -sulfate into rabbit articular cartilage was lower in 2 year old animals than in 3 to 5 month old animals. In vitro techniques gave similar results (79, 80) and were used to show that the incorporation of $[^{35}\text{S}]$ -sulfate into articular cartilage proteoglycans decreases with increasing age up to maturation after which it is relatively constant (79, 80, 71, 75, 5, 138).

Articular cartilage in osteoarthrosis

Definition. Osteoarthrosis, also called osteoarthritis, is at least initially, a noninflammatory disorder of diarthrodial joints. The earliest observable lesion is a focal disruption of the weight bearing surfaces of the articular cartilage. With time disintegration and abrasion of the whole thickness of the cartilage occur in large areas of the joint (18, 126). The diagnosis of osteoarthrosis in man is based on patient history and on clinical and radiological findings. The incidence of osteoarthrosis increases with increasing age (1) but it is uncertain whether advanced age is a prerequisite. During normal aging articular cartilage shows very limited morphological lesions mainly in non weight bearing areas (143, 20). Lesions however are not progressive and do not lead to symptomatic degeneration.

In about one fifth of patients the osteoarthrosis can be related to a preexisting condition generally of traumatic or inflammatory type (so called secondary osteoarthrosis), whereas in other cases no predisposing factor can be identified (so called primary osteoarthrosis) (18). Since little is known about the etiology of primary osteoarthrosis and since quite possibly also this entity is secondary (127, 128) to some factor not yet identified, the two forms are discussed together and when applicable a distinction is made between natural and experimental osteoarthrosis. A major problem in the study of human osteoarthrosis is, that many patients seek help late when the joint destruction is well advanced, and that the diagnostic methods, usually radiological, are crude and give little information in the early stages of the disease. A number of experimental models have therefore been developed in animals (see below) to study the early changes and hopefully provide a better understanding of the molecular mechanisms involved. Detailed descriptions of the pathology of osteoarthrosis may be found in reviews of Sokoloff (126) and Gardner (31).

Mechanical properties. In human osteoarthrotic cartilage the compressive stiffness as well as the tensile strength are lower than in normal articular cartilage (53, 54). As mentioned above the resistance to compression is mainly dependent on the content of glycosaminoglycans (37) while the collagen fibers determine the tensile properties of the cartilage (54). Thus the observed mechanical changes in osteoarthrosis indicate major alterations with regard to the two main macromolecular cartilage components, i.e. proteoglycans and collagen fibers.

Cartilage composition. Histological studies of osteoarthrotic cartilage show that an early event in the course of the disease is focal loss of metachromasia and stainability with cationic dyes, most noted near the surface. This implies a decreased content of glycosaminoglycans. In support chemical determinations of glycosaminoglycan contents have demonstrated marked losses of glycosaminoglycans and proteoglycans during development of cartilage degeneration (16, 15, 12, 132, 61, 91). In osteoarthrotic cartilage the content of CS is reduced (15, 46, 142) and the ratio of CS-6 to CS-4 is decreased (73, 87). The content of KS decreases both absolutely and as a proportion of total glycosaminoglycans, when compared to age matched normal cartilage (73, 132, 87).

In contrast to the marked changes in cartilage glycosaminoglycan composition and content, collagen content per dry weight does not change in osteoarthrosis (16, 72). Conflicting data suggest that the type of collagen in osteoarthrotic cartilage may (33, 96) or may not (26, 27, 30) be altered. It is, however, possible that the collagen fibers are affected. The increased thickness of osteoarthrotic cartilage together with the increased content of water, which is observed early in the degenerative process, could be indicative of a deranged collagen network or of impaired tensile properties of the collagen fibers (15, 74, 132, 142, 66).

Accordingly it has been suggested that fatigue of the collagen fibers because of excessive mechanical load is an important factor in the development of the lesion (29). Morphological evidence for collagen fragmentation as a late event in osteoarthritis has been presented (85, 29).

In addition to the molecular alterations in degenerating articular cartilage, the cell density increases progressively with time, at least in experimentally induced osteoarthritis (66). In human osteoarthrotic cartilage there are clusters of chondrocytes at the margins of fissures mainly near the cartilage surface (125). It has been suggested that this cell proliferation may be a reparative response to the loss of matrix in osteoarthritis (70).

Proteoglycan structure in natural osteoarthritis. In this section spontaneous osteoarthritis in humans and secondary degenerative changes in knee joints of dogs will be considered. A prerequisite for the isolation of pure and minimally degraded proteoglycans is the use of CsCl density gradient centrifugation as first described by Hascall & Sajdera (40). Therefore only studies using that technique will be reviewed when the structure of proteoglycans is discussed.

The extractability of proteoglycans from osteoarthrotic cartilage is increased (65, 64, 132). The higher extraction yields of the degenerated cartilage may result from the destruction of the collagen network and/or in situ fragmentation of the proteoglycans.

Proteoglycans isolated from degenerated areas of osteoarthrotic cartilage differ from proteoglycans from normal cartilage with respect to composition. A consistent finding is a lower relative KS content when determined as the ratio of glucosamine to galactosamine (10, 139). The proteoglycans from degenerated

human articular cartilage also have a lower protein content, which is manifested as a higher uronic acid to protein ratio (10).

The size of proteoglycan monomers from human degenerated femoral head articular cartilage, when compared to that of normal cartilage, has been reported to be decreased (139, 132). Other investigators (for refs. see 10), however, found no differences in proteoglycan hydrodynamic size.

The ability of proteoglycans to interact with HA was lower in human femoral head degenerate articular cartilage (139, 102). Furthermore, Oegema (97) observed that newly synthesized proteoglycans purified from slices of osteoarthrotic human femoral head articular cartilage incubated in vitro could not interact with HA, while those from control cartilage taken from normal femoral heads showed normal interaction. In tissue culture, however, the proteoglycans eventually acquired the capacity to interact with HA. Similarly, Harmand et al. (36) found that proteoglycans synthesized in culture by chondrocytes from human osteoarthrotic femoral heads had a somewhat reduced capability to aggregate with HA.

Taken together these data on proteoglycan structure and composition in natural osteoarthrosis indicate not only that the quantity of proteoglycans is diminished but that proteoglycan structure is modified. It seems that the proteoglycans are smaller, contain less KS and less protein and that some proteoglycan molecules have a defective HA binding region or lack it altogether. These proteoglycans may represent degradation products of the normal proteoglycans.

Proteoglycan structure in experimental osteoarthrosis. Studies on the development of osteoarthrosis at the molecular level in the spontaneous disease are hampered by large difficulties in the staging of the disease, selection of control specimen and sampling of pathological cartilage. Therefore much effort has been

devoted to the developement of experimental models in animals, where the duration of the disease is known and the different stages can be studied separately. Several experimental models in animals have been described (for complete references see 99). Most experimental models involve surgical manipulations of the knee joint, as this joint is easily accessible and in most animals a major weight bearing joint. In both humans and animals there is secondary knee joint osteoarthritis as a result of rupture of the anterior cruciate ligament. In the hip joint, two models have previously been described. One uses selectively bred Labrador dogs prone to severe spontaneous hip dysplasia with ensuing secondary osteoarthritis (89). The other model employs surgical myotomy to induce hip laxity and consequent osteoarthritis (107). Only very limited data on the structure and composition of proteoglycans are, however, available from either of these models. In the further discussion of experimental osteoarthritis only the surgically induced knee joint model of osteoarthritis will therefore be considered.

Several investigators have studied proteoglycan structure in knee joint cartilage degeneration induced by sectioning the anterior cruciate ligament in dogs (65, 134, 103) and in rabbits (24). The resulting unstable knee joint develops osteoarthritis within months. Knee articular cartilage degeneration has also been induced by sectioning of the meniscus (90, 122). One drawback with the surgical induction of knee joint instability is that the arthrotomy causes synovitis which may itself induce some of the early biochemical changes in the cartilage. Another limitation of the surgically induced knee joint instability models is that it does not fully progress to the developement of bony changes with total loss of cartilage and eburnation of bone (98). It seems that there is a definite proliferative element in this model, which may prevent the progression of cartilage degeneration to end stage osteoarthritis, presumably by increased reparative activity.

A consistent finding in induced osteoarthritis of the knee joint has been the loss of proteoglycans from the articular cartilage similar to that observed in the natural disease (64, 91). Furthermore, the remaining proteoglycans, also in the induced disease, show increased extractability. The relative content of CS in the proteoglycans increases compared both to KS (65, 64, 61) and protein while the size of the CS chains does not appear to change (61). The size distribution of the proteoglycans, in the early stages of the degeneration, is seemingly the same as that of proteoglycans from normal cartilage from the contralateral side or from sham operated joints (61, 91, 103) although supposedly degradation and elimination of the proteoglycans from the degenerated cartilage continuously occurs. The constant size of the proteoglycan monomers in the unstable knee joint may be explained by rapid clearance of degraded proteoglycans from the cartilage. The proteoglycans isolated from degenerated cartilage appear to have the same capacity to form aggregates as proteoglycans from control cartilage (62). The size of reconstituted native aggregates, however, was smaller (61, 134). Others (91, 103) found an impaired aggregation with HA.

In conclusion, the content of proteoglycans decreases in degenerated cartilage from unstable knee joints and although the size of the remaining proteoglycans is unchanged, their composition is markedly different, showing higher relative CS contents, compared to protein and KS contents. It may be that one of the subpopulations of the aggregating proteoglycans is preferentially lost from the tissue or that mainly one of the subpopulations is synthesised in response to the loss of matrix components.

The common finding in all forms of osteoarthritis is an early loss of proteoglycans and swelling of the cartilage. The composition of the proteoglycans is similarly changed in natural and experimentally induced osteoarthritis, i.e. the relative contents of CS is increased, while that of KS is decreased. It is possible that the underlying mechanism of cartilage destruction is

proteolytic fragmentation of the proteoglycan protein core and that the proteoglycan fragments are cleared from the cartilage at different rates in the different forms and various stages of the disease.

Metabolism of articular cartilage. Much effort has in the past been devoted to studies of the metabolic background to the marked changes in cartilage and proteoglycan composition in osteoarthritis. In most studies slices of articular cartilage have been incubated in radioactive medium containing precursors. A limitation of these studies is, however, that the cartilage piece has been removed from its natural environment and that the interactions with the surrounding tissues have been disrupted. One must therefore consider that data obtained from such systems may show results which are not physiologically relevant and that minor differences in culture conditions and stage of the disease may provide quite different results.

Proteoglycan synthesis in human osteoarthritis: With in vitro incubation techniques the incorporation of [^{35}S]-sulfate into proteoglycans in human femoral head and knee articular cartilage was found to be increased, relative to the contents of DNA or uronic acid (15, 76, 49, 135, 77). This indicates an increased proteoglycan synthesis in osteoarthrotic cartilage, maybe as a reparative response to the earlier loss of proteoglycans. In advanced osteoarthrotic degeneration the [^{35}S]-sulfate incorporation progressively decreased (76, 135) indicating that the process of reparation gradually ceases after a point of no return. Maroudas (79) correcting for diffusion time of isotope and McKenzie et al. (69), however, found no increased [^{35}S]-sulfate incorporation in proteoglycans in human osteoarthrotic femoral head cartilage.

Incorporation of [^3H]-glucosamine into proteoglycans in human hip osteoarthrotic cartilage in vitro was studied by Lipiello et al. (58) and Mankin et al. (77). No significant differences were noted in the incorporation of isotope into glucosamine as compared to galactosamine, suggesting that the observed relative decrease of KS in osteoarthrosis (cf. above) is not related to a qualitative alteration of proteoglycan synthesis.

Proteoglycan synthesis in experimental osteoarthrosis: In experimental dog knee osteoarthrosis the in vitro uptake of nondialyzable [^{35}S] sulfate per wet weight of cartilage is increased in the joint operated on compared to the contralateral joint. (103). McDevitt et al. (66) found that the newly synthesized proteoglycans in the osteoarthrotic cartilage were similar in size to those of the control cartilage and were capable of forming aggregates with HA (67). Using the same experimental model Palmoski & Brandt (103), however, reported that the newly synthesized proteoglycans in the degenerated cartilage could not form aggregates with HA. McDevitt et al. (67) also found evidence for an increased synthesis of CS relative to KS using in vivo labeling with [^{35}S]-sulfate. In support Floman et al. (27) also using in vivo labeling with [^3H]-glucosamine found a relative increase of [^3H]glucosamine incorporation into galactosamine compared to glucosamine during the first 2-5 weeks after operation (the time of transient synovitis induced by the operation) in samples of articular cartilage from any location in the operated joint. Later (16-40 weeks) after operation only areas with focal degeneration showed a consistent elevation of the ratio of [^3H]-galactosamine to [^3H]-glucosamine.

Although some conflicting data have been presented it appears from studies of experimental models employing the unstable knee joint that the rate of synthesis of CS compared to that of KS increases during the development of osteoarthrosis. It is possible that in this model the synthesis of only the CS-rich

subpopulation of aggregating proteoglycans increases in response to the loss of matrix molecules. Indeed Sandy et al. (118) reported that cartilage slices incubated *in vitro* responded to loss of cartilage matrix with synthesis of large, CS-rich proteoglycans.

Collagen synthesis. Available data from *in vivo* and *in vitro* studies, using labeling of collagen with [^3H]-proline, indicate that the synthesis of collagen is increased in both human hip osteoarthrotic cartilage and in experimental knee osteoarthrosis (57, 26, 27). The newly synthesized collagen was found to be type II collagen. Others, seemingly in contrast, have shown the presence of type I collagen in osteoarthrotic cartilage, probably synthesized in response to matrix changes (33). Yet other contrasting data are reported (89, 19) showing an accumulation of procollagen and a decreased *in vitro* and *in vivo* collagen synthesis in the degenerated hip articular cartilage of Labrador dogs selectively bred to develop osteoarthrosis secondary to hip dysplasia. The varying results may be due to differences in the species used and in the pathology of the cartilage studied.

DNA synthesis. The incorporation of [^3H]-thymidine per content of DNA, indicative of cell division, using *in vitro* incubation of human osteoarthrotic cartilage showed an increase with histological grade of osteoarthrosis up to a maximum at grade 10-11 whereafter it again decreased (76).

Mechanism for cartilage destruction. An early sign of osteoarthrosis is loss of proteoglycans, which may be due to proteolytic break-down, as discussed above. Several studies have shown the presence in cartilage and periarticular tissues of proteolytic enzymes capable of proteoglycan degradation. Increased activity of cathepsin D and neutral proteases as well as other lysosomal enzymes (2, 23, 123, 120, 117, 118, 47, 104) have been described

in osteoarthrosic cartilage. Furthermore, synovial membrane, particularly if inflamed, can produce factors like catabolin, which stimulate the chondrocyte to degrade the proteoglycans in its surrounding cartilage matrix (22). Sandy et al. (115) demonstrated that slices of normal rabbit articular cartilage incubated in vitro released slightly fragmented proteoglycans with decreased ability to bind to HA, probably as a result of limited proteolysis. The process required viable chondrocytes. It appears therefore that proteases and chondrocytes play a vital role in the turnover of cartilage macromolecules.

Although very variable results have been obtained, especially between different forms of osteoarthrosis of different severity, the above cited studies indicate drastic changes in the turnover of the various cartilage macromolecules. The studies indicate the presence in osteoarthrosis of degradative and reparative processes, which are unbalanced. Consequently the destruction of cartilage proceeds and the reparative process eventually fails. A complicating factor in interpreting the results is the variable synovitis, particularly in the surgically induced knee models of osteoarthrosis.

PRESENT INVESTIGATION

The aim of the present study was to provide information on the variation of proteoglycan composition in aging and in articular cartilage degeneration. Important background information was to be provided by studies of the variability of cartilage composition with distance from the articulating surface as well as in studies of intra-and inter-individual variability of cartilage composition.

Outline of analytical methods used

Frozen cartilage samples were powderized under liquid N₂, using either a Wiley mill or a rapid increase of pressure applied to two cooled metal blocks between which the cartilage sample were kept. Subsequent extraction was performed with 4M guanidinium chloride with or without proteinase inhibitors. The results were the same using either of the two solvents. Proteoglycans were then purified using equilibrium centrifugation in CsCl-gradients.

Glycosaminoglycan contents of proteoglycans were assayed by hexosamine analysis of the isolated glycosaminoglycan fraction, and oligosaccharide contents were determined by hexosamine analysis of the isolated oligosaccharide fraction.

The protocol used allows quantitation of the different types of glycosaminoglycans, particularly since HA can be quantified separately using a specific radioligand procedure.

Normal variation of articular cartilage proteoglycans (II, V)

Interindividual and intraindividual variation (V). The aim of this study was to determine the variation of cartilage and proteoglycan composition in the femoral head. The study would show whether or not in an experimental model of hip osteoarthro-

sis, the contralateral side, not interfered with, would provide an adequate control. Such information is presently lacking. To obtain normal cartilage greyhounds of racing lineage were used, as the incidence of hip dysplasia and osteoarthritis in these dogs is low. Normal, weight bearing articular cartilage was prepared fresh from the same localisation on the left and right side femoral head of 10 greyhounds 11-32 months of age. Analysis showed large variations between individuals with respect to most parameters measured, i.e. cartilage contents of proteoglycans, contents of HA, relative contents of CS, KS and protein in the proteoglycans. Size distributions of proteoglycan monomers and their aggregability with HA showed some variability between individuals, while size distributions of CS chains and extraction yields of proteoglycans varied little.

The intraindividual variation (the variation between the right and the left side femoral head articular cartilage) on the other hand was small with respect to all the parameters measured, i.e. those discussed above.

With regard to contents of proteoglycan subpopulations, agarose polyacrylamide gel electrophoresis showed similar electrophoretic mobilities and similar proportions of the proteoglycan subpopulations for the right and left side of the same individual. Between individuals the pattern could be quite different.

It can be summarized that the variation in proteoglycan composition and structure was small comparing the right and the left femoral head articular cartilage from the same individual. This justifies the use of the contralateral side as a control in experimental studies of joint disease, provided that only one joint is affected by the manipulations. The variations from one individual to another, with respect to the composition of the corresponding joint, are so large that even major changes induced by surgery may be obscured by this normal variability.

Variation with distance from the articular surface (II). The aim of this study was to provide information about the variability with distance from the surface of the proteoglycan composition within the weight bearing portion of the articular cartilage. For this study bovine articular cartilage was chosen, as the large dimensions of the bovine hip joint and the thickness of the articular cartilage allows analysis of samples from a small, well defined area.

The composition of the articular cartilage varied with increasing depth from the surface i.e. the content of glycosaminoglycans was highest in the middle portions of the articular cartilage. The most superficial, 40 μ m, layer of the articular cartilage was enriched in glucosaminoglycans. Except for this layer there was a gradual increase in glucosamine containing glycosaminoglycans from the surface towards the osteochondral junction. These results are in accordance with those reported by others (131, 83, 142, 10).

The yield of extraction was excellent for all sections of the cartilage and nearly all proteoglycans were extracted possibly due to the good fragmentation of the cartilage samples.

As expected the composition of the isolated proteoglycans varied with depth from the articular surface. The relative content of glucosamine showed a tendency to increase similar to that observed in extracts and cartilage. Part of the glucosamine content in the A1-fraction may be due to the presence of HA, especially in the deeper layers of the cartilage (25). Therefore, the change in content of glucosamine may at least partly be ascribed to changing contents of HA. The ratio of glucosamine to galactosamine in the proteoglycans was however generally higher than that of the glycosaminoglycans in extracts and cartilage. This was probably due to the presence of a large number of at the time unidentified glucosamine containing oligosaccharides in the proteoglycans. These oligosaccharides would separate from the glycosaminoglycan fraction in the ion exchange chromatography

used in the analysis of the extracts and cartilage. The relative content of glucosamine in the proteoglycans expressed per protein weight was relatively constant throughout the depth of the cartilage, although it showed a slight increase with distance from the articular surface. The relative CS content in the proteoglycans expressed as galactosamine/protein showed a similar variation as the total glycosaminoglycan content, i.e. being highest in the midportion of the cartilage. It was, however, again higher in the layer closest to the bone.

The most striking structural feature was the much smaller size of the proteoglycans in the top layer (200 μ m) of the cartilage. The proteoglycans in the deeper portions were of larger size and showed only minor size variations with further depth from the surface. These results are at variance with those of Bayliss & Venn (10) who found a progressive decrease in molecular size with increasing distance from the articular surface in a study of human articular cartilage. Species differences as well as differences in biological age may explain such seemingly contradictory results. Furthermore, the sampling of the cartilage is a variable which should be taken into account. In the present study proteoglycans were isolated separately from only one biopsy of weight bearing cartilage.

The ability of the isolated proteoglycans to interact with hyaluronic acid varied somewhat, in showing a slight increase of nonaggregating proteoglycans with increasing depth from the articular surface. These results are in accordance with those of Bayliss & Venn (10).

Agarose-polyacrylamide gel electrophoresis showed the presence of the two subpopulations of aggregating proteoglycans, although their relative proportions appeared rather constant. The slow migrating component increased somewhat with increasing depth from the surface.

In this context it should be stressed, that the observed compositional variation of the cartilage and the alterations in proteoglycan structure with depth from the articular surface, suggest that the changes observed in osteoarthritis do not simply represent wear of the superficial layers of the articular cartilage.

Structure of proteoglycans in aging cartilage (I,III)

Osteoarthritis is most common in the elderly person. It is likely, however, that also normal cartilage undergoes changes during aging. Therefore, this study was undertaken to demonstrate changes in proteoglycan composition and structure during aging. Two types of cartilage, having quite different function and load characteristics, were studied in order to provide information on the variability of the aging process and whether or not it may be related to functional demands on the tissue. Bovine tracheal cartilage was chosen as a hyaline cartilage that is non-weight bearing and which does not develop degenerative changes typical of osteoarthritis. Canine articular cartilage was chosen as a weight bearing cartilage which may develop cartilage changes typical of osteoarthritis.

The composition of proteoglycans from both the tracheal and the articular cartilage was found to vary markedly with age. Furthermore, the alterations were similar in the two types of cartilage.

There were, however, differences between the two tissues. Thus, the articular cartilage showed a somewhat decreased proteoglycan to collagen ratio, while in the tracheal cartilage the proteoglycan content increased slightly with increasing age. In the dog articular cartilage the ratio of KS to CS increased drastically with increasing age. This increase was less pronounced in tracheal cartilage and limited to the age period before maturation. The extraction yields of both tracheal and articular cartilage proteoglycans decreased markedly with age. The composi-

tion of the isolated proteoglycan varied with age in both cartilages, with the most notable changes occurring during maturation. Thus, the extracted proteoglycans were found to have decreasing contents of CS while contents of KS and protein increased with age.

In the study of the tracheal cartilage the composition of the proteoglycans was further characterized. The increasing content of CS-6 in aging agrees well with earlier studies (84, 86). The number of CS chains per molecule appear to decrease, while the number of KS chains increases during maturation. Both types of glycosaminoglycan chains remain relatively constant thereafter. The size of the KS side chains does not change appreciably. There was, however, a certain scatter of values and minor variations, especially during the period of maturation, could therefore not be excluded. The content of O-linked oligosaccharides varied inversely to that of the KS, i.e. they decreased in number during maturation and remained relatively constant with further aging. This may indicate that during maturation the O-linked oligosaccharides in newly synthesized proteoglycans are substituted to form KS, as also suggested by the results obtained by others (3, 112, 136, 116).

For both tracheal and articular cartilage proteoglycans the size of the CS chains appears to be essentially constant with increasing age, although there was a tendency for the chains of the articular cartilage proteoglycans to become somewhat smaller during maturation.

The proteoglycans become smaller with age, most markedly so with the articular cartilage proteoglycans. There were minor alterations in the ability of the proteoglycans to interact with HA. The tracheal cartilage proteoglycans, however, showed a somewhat decreased binding to HA in old age. For the articular cartilage proteoglycans the proportion of reconstituted aggregates in the A1-fraction was somewhat variable, but remained fairly constant in all ages.

The observed changes in composition and structure of the articular cartilage proteoglycan may in part be explained by a shift between the two proteoglycan subpopulations described by Heinegård et al. (45). Support was obtained in the studies of the tracheal cartilage. There the proportion of the CS-rich proteoglycans decreased relative to the contents of the KS-rich proteoglycans. A similar change to an increased proportion of a class of proteoglycans having a somewhat higher electrophoretic mobility was observed by Stanescu (129) studying baboon articular cartilage.

The alterations observed in normal aging are quite different to those seen in osteoarthritis. Therefore it appears that osteoarthritis does not simply represent a more pronounced aging process, but rather represents a distinct disease process.

Articular cartilage proteoglycans in osteoarthritis (I, IV).

Three types of osteoarthritis were studied:

I. Osteoarthritis secondary to congenital hip dysplasia was studied as an example of osteoarthritis occurring naturally in dogs. (paper III)

II. Experimentally induced hip joint osteoarthritis (paper IV). A new surgical model was used to induce osteoarthritis (99) by pelvic osteotomies, resulting in reduced acetabular coverage of the femoral head. Because of the extra articular nature of the surgical intervention, there is no accompanying synovitis.

III. Experimentally induced knee joint osteoarthritis (paper IV and VI). The most commonly used and biochemically best characterized (61) model of osteoarthritis, which is induced by transection of the anterior cruciate ligament in dogs, was studied as a reference to the experimental model of hip osteoarthritis (paper IV).

In the degenerated cartilage from the dogs with natural hip dysplasia, as well as in the cartilage from the joints operated on, the contents of glycosaminoglycans relative to collagen were generally reduced compared to that of control cartilage, although not statistically significantly so in the hip joints with natural hip dysplasia. In the cartilage of the hip joints operated on there was a significantly ($p < 0.05$) lower content of glycosaminoglycans per collagen compared with the contralateral control joint. Furthermore, there seemed to be a progressive loss of glycosaminoglycans from the cartilage with increasing time after surgery.

The content of KS relative to that of CS was not appreciably changed in the cartilage in the spontaneous disease nor in that from the hip joints operated upon.

The extraction yields, measured as recovery of glycosaminoglycans in extracts, was significantly higher in degenerated cartilage from the dysplastic hip joints as compared to normal control cartilage. Articular cartilage from the hip joints operated on surprisingly showed extraction yields similar to or lower than those of the control cartilage. The differences may be related to the different timespans for the development of the osteoarthritis, with relatively slowly developing cartilage degeneration in the dysplastic dogs and a much faster development in the experimentally induced osteoarthritis.

The proteoglycans isolated from the cartilage of the hips operated on had a higher content of CS while contents of KS and protein were lower compared with the contralateral joint. Furthermore, the size of the proteoglycans was reduced and their capacity to form aggregates with HA was very low even at short times after surgery. Similar changes were observed in the degenerated cartilage from the dogs with spontaneous hip dysplasia. These alterations in composition point at a degradation of the proteoglycans. Further support was obtained from studies of the composition of the extracts of cartilage from the induced femoral

head osteoarthritis. It was found that the extracts had a normal content of KS i.e. higher than the purified proteoglycans. It appears that some of this KS was located to fragments of proteoglycans which because of low contents of CS were lost to the upper portions of the CsCl-gradients used to purify the proteoglycans. The data, then, can be taken to indicate fragmentation of proteoglycans, probably by proteolysis.

The changes observed in the knee joints after transection of the anterior cruciate ligament differed in some aspects. Thus, the KS content relative to that of CS in the cartilage was lower at the sampling times longest after surgery, while the relative proportions of these glycosaminoglycans in the femoral head articular cartilage were unchanged. The lower extraction yields also observed with the degenerated knee cartilage were at variance with earlier observations by McDevitt et al. (65) and McDevitt & Muir (64), who had obtained higher extraction yields from the degenerated knee cartilage. In these studies, however, the normal cartilage showed very low extraction yields. It is possible that the "mincing" of the cartilage samples was more efficient in the present study allowing higher extraction yields of normal cartilage. In support it has been found that fragmenting the cartilage to small pieces is a prerequisite for good extraction yields (8).

Furthermore, the proteoglycans isolated from knee articular cartilage after transection of the anterior cruciate ligament showed much smaller structural changes. Although these proteoglycans showed compositional changes their size and aggregability appeared essentially constant. The data on the structure of the proteoglycans in the knee model are, however, similar to those published by McDevitt (61).

The discrepancy between the structural alterations in the secondary hip osteoarthritis to that of the experimental knee joint osteoarthritis may be explained by different rates of clearance of fragmented proteoglycans, by differences in the rate

of synthesis of proteoglycans, by differences in the induction of cartilage degeneration, by the presence of an inflammatory reaction in the operated knee joint or by other differences in the anatomy and pathophysiology of the two joints.

In summary, it may be noted that structural and compositional changes of the cartilage and proteoglycans from degenerated hip joint articular cartilage indicate that there is a degradation of the proteoglycans in osteoarthritis. The fragments produced are subsequently lost from the cartilage, possibly to the synovial fluid. The plausible reason for the structural alterations of the proteoglycan molecule is proteolytic degradation. The activity of proteolytic enzymes (cathepsins and neutral proteases) have been reported to be increased in osteoarthritis. However, selective degradation or selective synthesis of one of the proteoglycan subpopulations may produce similar changes in proteoglycan composition. McDevitt & Muir (64) have postulated increased synthesis of an immature type of proteoglycan rich in CS-4 and it appears that in the young animal there is a preferential synthesis of the large CS rich proteoglycan subpopulation. It is likely that in response to the loss of proteoglycans from the cartilage, the chondrocytes may respond by producing more proteoglycans.

Quantitation of synovial fluid proteoglycan antigens in experimental osteoarthritis (VI).

The changes of proteoglycans structure and quantity in induced osteoarthritis indicate a loss of molecules from the tissue, probably caused by increased proteolytic fragmentation of the core protein. Fragments produced can only diffuse to the synovial fluid. It should then be possible to quantitate release of such fragments provided that sensitive methods can be used. Examples of such methods are radioimmunoassay and enzyme linked immunosorbent assay (ELISA) that can be used if the fragments in the synovial fluid react with available antibodies. Thus, increased

amounts of proteoglycan antigens were detected with a newly developed ELISA studying the synovial fluid from the knees of German wirehaired pointers after transection of the anterior cruciate ligament. The synovial fluids from the joints operated on had significantly higher concentration of antigens related to the CS-rich subpopulation of aggregating proteoglycans compared with the synovial fluids from the contralateral side. Concentrations of antigens related to the KS rich subpopulation differed less. This difference in relative synovial concentration of the two proteoglycan subpopulations probably reflects different rates of release from the articular cartilage or alternatively may result from different rates of removal from the synovial cavity. It is, however, unlikely that the source of the proteoglycan antigenic structures was tissues other than the articular cartilage. Data presented on other cartilages may provide some insight into the nature of the difference. Thus Triphans et al. (138) in metabolic experiments found lower specific activity of the KS in KS-rich proteoglycans relative to the KS in CS-rich proteoglycans. Furthermore, the turnover rate for keratan sulfate is slower than that of CS (for ref. see 93). It thus appears that the normal turnover of the two populations of proteoglycans may be rather different, that of the KS rich being slower.

During the course of development of the degeneration the concentration of proteoglycan antigens in the synovial fluid samples from an individual joint was higher during the postoperative period. Again the changes were most marked for the antigens related to the CS-rich proteoglycans. Some of the proteoglycan antigens may be released in an inflammatory response to the arthrotomy. It is known that leukocytes and macrophages produce enzymes capable of cartilage degradation (for refs. see 21). Furthermore, the articular cartilage chondrocytes may be stimulated to degrade cartilage by the release of catabolin from the synovium or from lymphocytes (113).

It is likely that the release of antigens from the affected cartilage is much underestimated, since only concentrations were measured. All dogs had marked hydrops only of the joint operated on. It is therefore likely that the total amount of antigen in this joint is higher compared to that of the control joint, than what is indicated by the concentrations.

This newly developed method may be of importance for future work on osteoarthritis, as it may offer an opportunity for an early diagnosis. The method may also be useful in the future evaluation of therapy.

CONCLUDING REMARKS

The biomechanical properties of articular cartilage are dependent on the integrity of the cartilage macromolecules, mainly of the proteoglycans and the collagen, and on the viability of the chondrocytes. Any derangements in one or more of these components will cause loss of mechanical function. This will increase the demands put on the cartilage by normal or excessive weight bearing, which may cause damage to the cartilage matrix and/or chondrocytes. Impaired mechanical function may thus perpetuate any primary damage to the cartilage and cause the development of cartilage degeneration, as in osteoarthritis. The primary event in the process is unknown, but it is probable that mechanical factors play an important part in the development of primary osteoarthritis.

The structure of hyaline cartilage proteoglycans changes similarly with increasing age in both tracheal and articular cartilage. The compositional and structural changes observed in aging differ from those observed in cartilage degeneration. The marked decrease in the content of proteoglycans in the articular cartilage is typical of osteoarthritis. Furthermore, the data indicate that this is due to an increased degradation of proteoglycans. Attempts by the tissue at repair or remodeling in response to increased functional demands may show as an increased synthesis. Eventually the repair process may fail, possibly because of excessive mechanical load. Cartilage degeneration proceeds to cartilage erosion and to loss of cartilage in the end stages of osteoarthritis.

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APPENDIX
papers I-VI

Articular-Cartilage Proteoglycans in Aging and Osteoarthritis

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The composition of macroscopically normal hip articular cartilage obtained from dogs of various ages was studied. Pieces of cartilage with signs of degeneration were studied separately. In normal aging, the extraction yield of proteoglycans decreased; the keratan sulphate content of extracted proteoglycans increased and the chondroitin sulphate content decreased. The extracted proteoglycans were smaller in the older cartilage, mainly owing to a decrease in the chondroitin sulphate-rich region of the proteoglycan monomers. The hyaluronic acid-binding region and the keratan sulphate-rich region were increased and the molar concentration of proteoglycan probably increase with increasing age. The degenerated cartilage had higher water content and the proteoglycans, as well as other tissue components, gave higher yields. The proteoglycan monomers from the degenerated cartilage were smaller than those from normal cartilage of the same age, and hence had a smaller chondroitin sulphate-rich region and some of the molecules also appeared to lack the hyaluronic acid-binding region. Increased proteolytic activity may be involved in the process of cartilage degeneration.

Articular cartilage contains few cells and an abundance of surrounding extracellular matrix, which consists mainly of collagen and proteoglycan. The main components contributing to the elasticity of the articular cartilage are the proteoglycans (for references see Kempson, 1975), which are composed of a central protein core to which a large number of highly negatively charged polysaccharide chains of chondroitin sulphate and keratan sulphate are covalently attached. At one end, the hyaluronic acid-binding region (see Fig. 1), about one-third of the protein core forms a globular structure (Heinegård & Hascall, 1974a) to which few or no polysaccharide chains are attached. This region is capable of interacting specifically with hyaluronic acid. Several individual proteoglycan monomers can bind to one hyaluronic acid molecule to form large aggregates. In normal cartilage a large proportion (80–90%) of the proteoglycans are able to form aggregates. The major portion of the keratan sulphate chains and a few chondroitin sulphate chains are attached to the keratan sulphate-rich region (see Fig. 1) (Heinegård & Axelsson, 1977). The remaining 90% of the chondroitin sulphate chains and some keratan sulphate chains are attached to a third portion of the protein core, the chondroitin sulphate-rich region (see Fig. 1) (Heinegård & Axelsson, 1977). The chondroitin sulphate-rich region is probably essential for the resilience of the proteoglycans, because it contains a large number of negatively charged groups.

The elasticity of cartilage is largely related to the

content and the structure of the proteoglycans in the matrix (Harris *et al.*, 1972; Scott, 1973, 1975). Kempson *et al.* (1971) showed that the elasticity of osteoarthritic cartilage is decreased. It is therefore likely that the composition of the proteoglycans has changed.

Primary osteoarthritis is usually a disease of old age in both man and animals. In contrast, osteoarthritis that is secondary to disease or trauma often occurs in young and middle-aged individuals. In secondary osteoarthritis, the macroscopic and histological changes in the joint cartilage are similar to those found in osteoarthritis. Hip dysplasia with secondary osteoarthritis is common in certain breeds of dogs; such dogs could therefore be a readily available source for studying changes of the proteoglycan composition in degenerated cartilage. A prerequisite for better understanding of the changes of cartilage composition in osteoarthritis, however, is a knowledge of the normal changes with age.

In pig articular cartilage the glucosamine/galactosamine ratios increased with age (Šimůnek & Muir, 1972). This implies that the content of keratan sulphate compared with chondroitin sulphate is relatively higher in the articular cartilage from the older animal.

It has been suggested that degradation of the proteoglycans is an important step in the development of osteoarthritis. Accordingly Ali & Evans (1973) reported an increased activity of cathepsins in osteoarthritis with a concomitant loss of uronic acid,

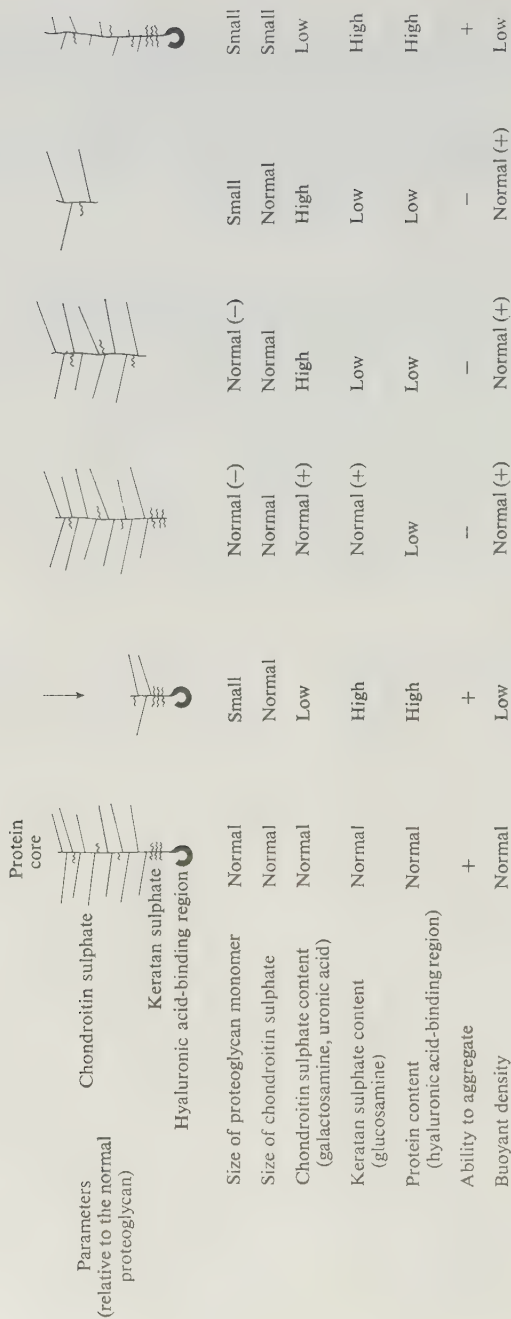


Fig. 1. Structure and composition of proteoglycan monomers after conceivable modifications. The structure proposed for proteoglycans from aging cartilage is indicated with an arrow. (The normal structure is to the left, with all parameters indicated as normal.)

probably because of degradation of the proteoglycans. McDevitt *et al.* (1973) reported an increased ratio of galactosamine/glucosamine in proteoglycans isolated from osteoarthritic knee-joint cartilage of dogs. The osteoarthritis had been induced by cutting the anterior cruciate ligament in the knee joint. Altman *et al.* (1973) have reported decreased uronic acid/protein ratios in proteoglycans from osteoarthritic cartilage.

In summary, available data indicate that the proteoglycan structure is changed in osteoarthritis, although seemingly contradictory results have been obtained.

Work on the structure of cartilage proteoglycans indicated that protein, keratan sulphate and chondroitin sulphate are asymmetrically distributed in the proteoglycan molecules (see Fig. 1) (Heinegård & Hascall, 1974a; Heinegård & Axelsson, 1977). Therefore changes in the relative proportions of the polysaccharides and protein can be used as markers of changing size of the proteoglycans, and also to indicate the mechanism behind these changes. Direct measurements of the size of the proteoglycans will give supporting information.

The aim of the present investigation was primarily to establish whether there are changes in the quantity and structure of the proteoglycans of aging articular cartilage, and secondarily to establish whether an altered proteoglycan structure in the degenerated cartilage of osteoarthritis (when compared with normal cartilage of the same age) could explain the decreased elasticity of the cartilage.

Materials

Fresh articular cartilage was obtained within 5 min after death from the femoral heads of ten dogs, aged 4.5 months–13 years (eight German Shepherd dogs and two Afghan Hounds, both the latter aged 5 years). From eight of the dogs, both apparently normal cartilage and degenerated cartilage was obtained. Cartilage was considered degenerated when it was discoloured, had lost its normal glossiness or was eroded. In all dogs with degenerated cartilage, hip dysplasia was the cause of osteoarthritis. In three dogs (age 6, 8 and 12 months) normal cartilage was taken from animals with osteoarthritis of one hip only, and in these cases the normal cartilage was taken from the normal contralateral hip joint. From five animals (aged 0.5, 5, 5, 8 and 10.5 years) cartilage with normal appearance was taken from the same joint as the degenerated cartilage. From one of these dogs, aged 6 months, apparently normal cartilage was obtained both from a joint that had focal cartilage degeneration and from the apparently normal contralateral joint. The youngest dog (aged 4.5 months) had subluxation of both hip joints, and 19 days before it was killed it had been operated on. Pelvic osteotomy was

performed on one side in an effort to minimize subluxation. The operation was a failure and actually caused degenerative changes to appear in the joint cartilage. All the cartilage in the contralateral joint had a normal appearance. One dog, aged 17 months, had normal cartilage in both hip joints and a sample was taken only from one joint. Only degenerated cartilage could be obtained from one dog, aged 13 years, which had advanced osteoarthritis of both hip joints.

All samples of degenerated cartilage were taken from the small semilunar area dorsal to ligamentum teres, which is a major weight-bearing part of the femoral head. Cartilage samples from normal joints were taken from the same location on the joint surface. In some cases apparently normal cartilage was taken from the dorsal aspect of the femoral head, which contained areas showing degeneration. Since all cartilage samples were prepared from the dorsal and most weight-bearing area of the femoral head, it was considered that differences in the structure of the proteoglycans would not be due to variations in the site from which the macromolecules were prepared.

In all cases, the cartilage was classified as being either normal (whitish-blue with glossy surface) or degenerated. Four grades of degeneration were recognized: (a) discoloured cartilage with glossy surface; (b) fibrillated cartilage with loss of normal glossiness; (c) eroded cartilage; (d) severely eroded cartilage adjacent to defects with denuded subchondral bone.

Cartilage samples were dissected, wiped clean with a paper napkin, cut into small pieces (approx. 2 mm in diam.) and frozen in liquid N₂. The frozen samples were then immediately put into a stainless-steel cylindrical chamber (diam. 10 mm, depth 32 mm), precooled in liquid N₂. A stainless-steel cylindrical piston (diam. 9.5 mm, height 61 mm), also precooled in liquid N₂, was then fitted into the chamber and the cartilage was powdered by applying a pressure of 196 MPa to the piston for 2 s. The cartilage powder was stored at -30°C until extraction.

Methods

Extraction

Samples (9.1–230.8 mg) of cartilage powder from the various dogs were extracted in 3 ml of 4 M-guanidinium chloride/0.05 M-sodium acetate, pH 5.8, for 24 h at 3°C (Hascall & Sajdera, 1969; Heinegård, 1972). Extracts were separated from residues by centrifugation at 21 700 *g* (*r*_{av}, 8.6 cm) for 20 min in a Sorvall RC 2-B centrifuge with the SE-12 rotor at 4°C. The residues were resuspended in 0.5 ml of 4 M-guanidinium chloride/0.05 M-sodium acetate, pH 5.8, and again centrifuged at 22 700 *g* (*r*_{av}, 9.0 cm) for 20 min. The supernatants were added to the respective extracts discussed above. This procedure was

repeated twice to assure complete separation of extracts from residues.

Associative-density-gradient centrifugation (Hascall & Sajdera, 1969; Heinegård, 1972)

The concentration of guanidinium chloride in the extracts was brought to about 0.4 M by dialysis of the extracts against 9 vol. of 0.05 M-sodium acetate, pH 5.8, for 24 h at 3°C. The densities were subsequently adjusted to 1.61–1.62 g/ml by the addition of solid CsCl. The solutions were centrifuged in an MSE preparative ultracentrifuge in a 10 × 10 ml aluminium angle rotor at 34000 rev./min (83000g, r_{av} , 6.46 cm) for 60 h at 12°C. Immediately after centrifugation the tubes were placed vertically in a stand and frozen by immersion in liquid N₂. The frozen tubes were then sawn in two (by using a guide) to separate the bottom 40% (by vol.) of the gradient (fraction A1) from the top (fraction A2). Densities of the bottom and the top fractions were measured by pycnometry with a 250 μ l constriction pipette.

Chemical methods

Protein contents of fraction A1 were determined by the micro-procedure of Lowry *et al.* (1951) after the samples had been dialysed against sodium acetate and water. Uronic acid in both fractions A1 and A2 were measured by the procedure of Bitter & Muir (1962), but with one-tenth volumes. Hydroxyproline contents of cartilage samples were determined as described by Stegemann & Stalder (1967) after hydrolysis of samples in 6M-HCl in sealed tubes for 24 h at 100°C.

Keratan sulphate and chondroitin sulphate contents of extracts and residues were determined on samples digested with papain, and the glycosaminoglycans were isolated by ion-exchange chromatography as described by Axelsson & Heinegård (1975). The samples were then hydrolysed in 6M-HCl for 8 h in sealed tubes at 100°C and glucosamine and galactosamine were separated and determined with an automatic amino acid analyser. Galactosamine contents of the glycosaminoglycan fractions are proportional to the contents of chondroitin sulphate, whereas glucosamine contents of the glycosaminoglycan fractions are approximately proportional to the contents of keratan sulphate. Hyaluronic acid, however, also contains glucosamine, but, since the quantities of hyaluronic acid in cartilage is very small (Hardingham & Muir, 1973; Hascall & Heinegård, 1974), its contribution to the glucosamine content is minimal and will be disregarded. Glycosaminoglycans of extracts and residues were calculated as the sum of glucosamine and galactosamine in the glycosaminoglycan fractions.

The proportions of aggregates and monomers in fraction A1 were determined by gel chromatography

on Sepharose 2B. The excluded peaks represent the aggregates (Heinegård, 1972).

To determine representative differences in the size distribution of the proteoglycan monomers in fraction A1, the ability of the monomers to interact with hyaluronic acid was first inhibited by reduction and alkylation of the preparations (Heinegård, 1977). Such a treatment destroys the tertiary structure and the function of the hyaluronic acid-binding region by breaking the disulphide bridges. The samples were then chromatographed on Sepharose 2B, to yield the elution profiles of the proteoglycan monomers. The size distribution of the chondroitin sulphate side chains of the proteoglycans in fraction A1, was determined after the samples had been digested with papain (5 μ g/mg of proteoglycan) in 0.05 M-sodium phosphate buffer/5 mM-cysteine/5 mM-EDTA, pH 6.5 at 65°C, for 3 h. The samples were then chromatographed on Sephadex G-200 (Wasteson, 1971).

Gel chromatography

Sepharose 2B and Sephadex G-200 gel chromatography was performed on columns (about 140 cm × 0.6 cm) eluted with 0.5 M-sodium acetate, pH 7.0, at a constant temperature of 4°C. Constant flow of eluent (1.5 ml/h) through the columns was obtained by using a constant-flow-rate infusion pump equipped with a 50 ml plastic syringe. Fractions (0.89 ml) were collected with a time-controlled fraction collector and analysed for uronic acid content by an automated version of the carbazole method (Heinegård, 1973).

Calculations

The analytical values obtained with normal and osteoarthritic cartilage from the same individuals were compared by Student's *t* test for paired observations. The youngest dog (aged 4.5 months) was not included in the *t* test, as it had bilateral subluxation of the hip. Linear-regression correlation coefficients for values from all cartilage, excluding the degenerated cartilage from the operated joint of the youngest dog, were calculated by the method of least squares. Age was correlated to the chemical data obtained both with the normal and the degenerated cartilage. A Student's *t* test (Rao, 1965) was performed on each regression line from the normal cartilages to test the hypothesis that the line does not deviate from horizontal, i.e. that the chemical parameters remain constant through all age groups.

Results and Discussion

Normal cartilage

Composition. Glycosaminoglycans (per wet weight or per hydroxyproline content of whole cartilage) showed a small decrease with increasing age, although

the linear correlation coefficients were low (Table 1). Hydroxyproline (per wet weight of cartilage) showed no significant variation with age (Table 1). The keratan sulphate content of cartilage showed a significant increase with age when expressed as percentage of glycosaminoglycans (correlation coefficient 0.89, $P<0.001$), but also when related to hydroxyproline content or wet weight of cartilage (Table 2). The relative keratan sulphate content increased more in the cartilage from the youngest dogs, but as discussed the oldest contained more keratan sulphate.

The amounts of keratan sulphate compared with chondroitin sulphate in cartilage increase with age (Mathews & Glagov, 1966; Šimůnek & Muir, 1972), and our data corroborate these findings. However,

the absolute amount of keratan sulphate in the articular cartilage increases with age and there is only a minor decrease in contents of total cartilage glycosaminoglycans with age.

Extraction. In the present work proteoglycans were extracted with guanidinium chloride without the proteinase inhibitors used by Oegema *et al.* (1975). It should be noted, however, that all procedures were done in the cold and in a direct sequence. Therefore it is unlikely that any degradation of the macromolecules occurred during extraction. Cartilage proteoglycans prepared from guanidinium chloride extracts with and without proteinase inhibitors have the same structure and composition (D. Heinegård, unpublished work). It should also be pointed out that

Table 1. Composition of normal and osteoarthritic cartilage

Extraction yields and tissue contents of glycosaminoglycans are related to wet weight and hydroxyproline contents.

Normal cartilage	Age (months)	Hydroxy- proline per wet wt. of cartilage (%)	Extraction yield [% of polysaccharide hexosamine (extract/ tissue)]	Glycosaminoglycan contents					
				Glycosaminoglycan hexosamine/wet wt. cartilage (µg/mg)			Glycosaminoglycan hexosamine/hydroxyproline in tissue (µg/µg)		
				Extract	Residue	Tissue	Extract	Residue	Tissue
	4.5*	5.18	89.3	34.4	3.9	38.3	0.662	0.079	0.741
	4.5	2.72	80.6	16.8	4.1	20.9	0.625	0.150	0.775
	6	4.84	81.9	22.6	5.0	27.6	0.467	0.102	0.569
	6*	3.43	82.4	15.4	3.2	18.6	0.449	0.095	0.544
	8	4.20	75.9	12.7	3.9	16.7	0.303	0.095	0.397
	12	2.54	76.8	7.2	2.2	9.3	0.283	0.084	0.367
	17	4.19	76.2	21.7	6.8	28.5	0.517	0.161	0.678
	60*	4.28	67.8	10.7	5.2	15.9	0.251	0.121	0.372
	60*	3.36	69.9	17.9	7.5	25.4	0.532	0.227	0.759
	96*	3.66	65.1	10.4	5.6	15.9	0.210	0.165	0.435
	126*	3.28	56.6	10.6	7.9	18.4	0.320	0.245	0.566
Mean	36	3.79	74.8	16.4	5.0	21.4	0.420	0.139	0.564
Correlation coefficient		-0.20	-0.93	-0.45	0.69	-0.28	-0.52	0.77	-0.18
P (regression line)		>0.4	<0.001	<0.2	<0.02	<0.4	<0.1	<0.01	>0.4
Osteoarthritic cartilage									
Classified group									
a	4.5	3.45	72.1	6.4	2.5	9.0	0.188	0.073	0.261
a	6	3.14	82.0	16.5	3.6	20.0	0.523	0.115	0.637
b	8	4.45	88.8	18.6	2.3	20.9	0.417	0.052	0.469
b	12	1.87	78.9	8.1	2.1	10.2	0.426	0.115	0.541
c	60	2.76	70.6	6.4	2.7	9.1	0.232	0.098	0.330
c	60	3.07	72.4	7.0	2.7	9.7	0.227	0.086	0.313
c	96	2.40	73.6	11.1	3.9	15.0	0.462	0.166	0.628
b	126	3.32	63.6	7.7	4.5	12.2	0.233	0.134	0.367
d	156	2.87	75.6	17.4	5.5	22.9	0.603	0.195	0.797
Mean	59	3.04	75.3	11.0	3.3	14.3	0.368	0.115	0.483
Correlation coefficient		-0.14	-0.67	-0.09	0.85	0.10	0.04	0.77	0.24
P (normal versus osteoarthritic)		<0.05	<0.025	<0.3	<0.01	<0.2	>0.4	>0.2	>0.4

* Apparently normal cartilage from joint with arthritic changes.

Table 2. *Composition of normal and osteoarthritic cartilage*
 Contents of chondroitin sulphate and keratan sulphate are shown in extracts, extraction residues and tissue.

Normal cartilage	Age (months)	Keratan sulphate/ chondroitin sulphate (glucosamine as % of polysaccharide hexosamine)			Keratan sulphate contents					
					Glycosaminoglycan glucosamine/wet wt. cartilage ($\mu\text{g}/\text{mg}$)			Glycosaminoglycan glucosamine/hydroxyproline in tissue ($\mu\text{g}/\mu\text{g}$)		
		Extract	Residue	Tissue	Extract	Residue	Tissue	Extract	Residue	Tissue
	4.5*	5.7	7.4	5.9	1.95	0.30	2.26	0.038	0.005	0.043
	4.5	8.9	4.3	8.0	1.50	0.16	1.66	0.055	0.007	0.063
	6	12.9	5.9	11.7	2.92	0.30	3.22	0.061	0.005	0.066
	6*	9.3	6.1	8.7	1.43	0.20	1.63	0.041	0.007	0.048
	8	11.2	8.4	10.5	1.41	0.34	1.75	0.034	0.007	0.041
	12	17.6	9.9	15.8	1.27	0.20	1.47	0.050	0.009	0.059
	17	22.7	14.9	20.9	4.92	1.15	6.07	0.118	0.023	0.141
	60*	26.2	18.3	23.7	2.81	0.93	3.74	0.066	0.022	0.087
	60*	28.7	20.4	26.2	5.12	2.49	6.68	0.152	0.047	0.199
	96*	24.2	19.9	22.7	2.51	1.11	3.62	0.068	0.030	0.098
	126*	39.9	26.8	34.2	4.19	2.15	6.34	0.127	0.066	0.193
Mean	36	18.9	12.9	17.1	2.73	0.76	3.49	0.074	0.021	0.094
Correlation coefficient		0.89	0.94	0.89	0.50	0.78	0.64	0.59	0.90	0.71
<i>P</i> (regression line)		<0.001	<0.001	<0.001	<0.2	<0.01	<0.05	<0.1	<0.001	<0.02
Osteoarthritic cartilage										
Classified group										
a	4.5	6.8	9.8	7.7	0.45	0.25	0.70	0.013	0.007	0.020
a	6	13.6	16.9	14.2	2.24	0.61	2.85	0.070	0.020	0.090
b	8	6.7	7.6	6.8	1.25	0.18	1.43	0.027	0.004	0.030
b	12	20.6	17.1	19.9	1.65	0.36	2.00	0.088	0.020	0.107
c	60	24.3	21.9	23.6	1.56	0.59	2.15	0.057	0.021	0.078
c	60	30.6	21.9	28.2	2.13	0.59	2.72	0.068	0.020	0.088
c	96	24.3	22.7	23.8	2.69	0.91	3.60	0.111	0.038	0.149
b	126	32.9	32.9	32.8	2.56	1.45	4.01	0.077	0.043	0.120
d	156	32.8	27.8	31.6	5.67	1.58	7.25	0.199	0.054	0.252
Mean	59	21.4	19.8	21.0	2.24	0.72	2.97	0.079	0.025	0.104
Correlation coefficient		0.83	0.86	0.85	0.79	0.94	0.86	0.73	0.93	0.80
<i>P</i> (normal versus osteoarthritic)		>0.4	<0.025	>0.4	<0.1	<0.2	<0.2	>0.4	>0.4	>0.4

* Apparently normal cartilage from joint with arthritic changes.

any proteolytic cleavage of the proteoglycan molecules, resulting in peptide liberation, would tend to decrease the protein content of fraction A1, in contrast with observations for aging cartilage (see below).

The proportion of the proteoglycans that could be extracted with 4M-guanidinium chloride decreased with increasing age (Table 1). Similar results have been obtained with pig articular (Šimůnek & Muir, 1972) and bovine tracheal cartilage (Larsson & Heinegård, 1975). The decreased ease of extraction of proteoglycans with increasing age cannot be readily explained at present since the structural or functional difference between extracted and residual proteoglycans is obscure. It should be noted (see below) that the proteoglycans that were not extracted contained rela-

tively less keratan sulphate compared with the molecules extracted at each age (Table 2), which may indicate structural differences. Further, the content of keratan sulphate of extracted proteoglycans showed a regular increase with age, the linear regression correlation coefficient being 0.89 and $P < 0.001$. This finding agrees with results of others (Mathews & Glagov, 1966; Šimůnek & Muir, 1972). The relative proportion of keratan sulphate in the glycosaminoglycans of the residues also increased with increasing age (Table 2), all values, however, being lower than those of the extracted proteoglycans of the same age.

Associative-density-gradient centrifugation. The content of uronic acid in the fraction A1 is proportional to the chondroitin sulphate and therefore

Table 3. *Composition of normal and osteoarthritic cartilage*

Relative distribution and contents of chondroitin sulphate (uronic acid) and protein are shown in fractions from associative CsCl-density-gradient centrifugation.

Normal cartilage	Age (months)	Chondroitin sulphate/ protein (uronic acid/ protein in fraction A1)	Uronic acid (% of dry wt.)		Protein in fraction A1 (% of dry wt.)	Uronic acid in fraction A1 (% of total uronic acid in gradient)
			Fraction A1	Fraction A2		
	4.5*	1.24	16.5	9.0	13.2	69
	4.5	1.75	24.0	8.1	13.7	84
	6	1.25	14.0	9.2	11.2	68
	6*	1.35	24.4	6.5	18.1	86
	8	1.60	30.4	8.6	19.0	77
	12	0.89	11.8	7.6	13.2	77
	17	1.10	22.5	8.2	20.4	83
	60*	0.98	13.9	9.9	14.2	65
	60*	0.72	20.3	7.7	28.1	83
	96*	1.00	20.3	8.9	20.3	68
	126*	0.82	17.3	9.1	21.0	61
Mean	36	1.16	19.6	8.4	17.5	75
Correlation coefficient		-0.65	-0.20	0.38	0.51	-0.59
P (regression line)		<0.05	>0.4	<0.3	<0.2	<0.1
Osteoarthritic cartilage						
Classified group						
a	4.5	1.44	15.9	6.8	11.1	58
a	6	1.69	23.0	5.1	13.6	78
b	8	1.93	26.6	4.4	13.8	85
b	12	1.11	20.0	6.3	18.1	86
c	60	1.71	21.9	6.9	12.8	66
c	60	0.77	12.0	6.7	15.4	61
c	96	1.21	23.2	7.7	19.3	81
b	126	1.08	17.0	5.0	15.8	78
d	156	0.78	17.7	10.3	22.7	62
Mean	59	1.30	19.7	6.6	15.8	73
Correlation coefficient		-0.64	-0.45	0.67	0.65	-0.50
P (normal versus osteoarthritic)		<0.01	>0.4	<0.005	<0.3	<0.4

* Apparently normal cartilage from joint with arthritic changes.

to the proteoglycan content of this fraction. The chondroitin sulphate (uronic acid) content of the fraction A1 expressed as percentage of the total chondroitin sulphate in the gradient decreased with increasing age of the cartilage (Table 3). Proteoglycans that have a lower buoyant density in a CsCl gradient usually are smaller and have a relatively higher protein content and a lower chondroitin sulphate content (Heinegård, 1977; Rosenberg *et al.*, 1976). Therefore the distribution of proteoglycans in the gradient (Table 3) indicated that the proportion of proteoglycans with higher protein content (i.e. smaller molecules) increases with age. Table 3 also shows that the protein content of fraction A1 increases with increasing age, indicating a higher protein content of the proteoglycans. Since the protein content of the proteoglycans is largely dependent on the hyaluronic acid-binding region, it is likely that the relative content of the latter increases

with increasing age. The relative uronic acid content of fractions A1, however, showed only a minor decrease with increasing age. Such a seemingly contradictory result could simply be explained by the fact that the chondroitin sulphate chains (uronic acid) are by far the major component of the proteoglycans and that small differences in the chondroitin sulphate contents may be technically difficult to discern.

The spacing of chondroitin sulphate chains along the polysaccharide-binding region of the protein core is considered to be similar for proteoglycans of all sizes (Heinegård & Hascall, 1974b; Heinegård, 1977; Heinegård & Axelsson, 1977; Thyberg *et al.*, 1975). Therefore the number of chondroitin sulphate chains is probably determined by the relative length of this region of the core. It is assumed in the present work that degenerated or aging cartilage has the same spacing of chondroitin sulphate chains as the normal cartilage. The decreasing uronic acid/

protein ratio (Table 3) of fraction A1 with increasing age, then, indicates that the chondroitin sulphate-binding region of the molecule [containing little protein (Heinegård & Axelsson, 1977)] becomes smaller with increasing age, assuming that the hyaluronic acid-binding region is intact (see below).

The glucosamine/galactosamine ratio (keratan sulphate/chondroitin sulphate) of the extracted proteoglycans increased with increasing age (Table 2). The major portion of the keratan sulphate is located in the keratan sulphate-enriched region between the hyaluronic acid-binding region and the chondroitin sulphate-enriched region (Heinegård & Axelsson, 1977). It appears, then, that proteoglycans from older cartilage contain a relatively higher proportion of the keratan sulphate-enriched region.

The increased content of the hyaluronic acid-binding region and of the keratan sulphate-enriched region, compared with the chondroitin sulphate-enriched region of the proteoglycans from the older cartilage, suggests that the size of the molecules decreases with increasing age (cf. Fig. 1).

Gel chromatography. All cartilage fraction-A1 preparations contain variable proportions of proteoglycan aggregates and non-aggregated proteoglycan monomers (Hascall & Sajdera, 1969; Heinegård, 1972; Hascall & Heinegård, 1974). Sepharose 2B gel chromatography (Heinegård, 1972) is the technically most simple method to determine the proportion of non-aggregated proteoglycans in fraction A1 isolated from cartilage (Fig. 2). Only minor differences can be seen between the various chromatograms. The proportion of the non-aggregated monomer proteoglycans (included peak, Fig. 2), however, is somewhat larger in the very youngest cartilage. The results suggest that the capability of the proteoglycans to bind to hyaluronic acid is not significantly changed in the older cartilage and therefore it is likely that the same proportion of the molecules from all age groups contain the hyaluronic acid-binding region.

The size of the proteoglycan monomers in fraction A1 was determined by Sepharose 2B chromatography. To prevent aggregation the proteoglycans were reduced and alkylated in 4M-guanidinium chloride before chromatography on Sepharose 2B (Heinegård, 1977; Fig. 3). The advantage of this reduction technique compared with conventional CsCl-density-gradient centrifugation is that it accounts for all the monomers in fraction A1, whereas the density gradient selects larger monomers (Heinegård, 1977). The older the cartilage the more retarded were the proteoglycan monomers eluted (Fig. 3). Therefore the size of the proteoglycan monomers gradually decreased with increasing age of the cartilage.

Fractions A1 were digested with papain and chromatographed on Sephadex G-200 to reveal the

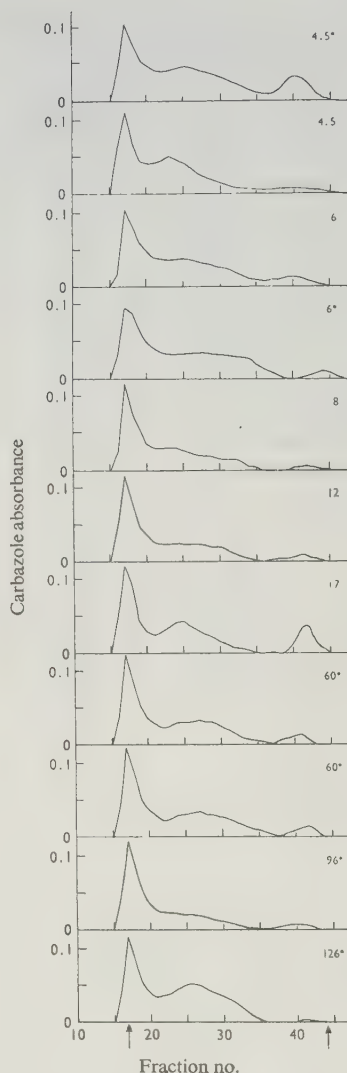


Fig. 2. Sepharose 2B column (1400 mm $\times$ 6 mm) chromatograms of fraction A1 from the associative-density-gradient centrifugation from normal cartilage

The size of the peaks is corrected so that the area under the void volume peak is constant. The void volume and total volume are indicated by arrows. The age of the dog (in months) is given on each part of this and subsequent Figures. *Normal cartilage obtained from joint with degenerative changes.

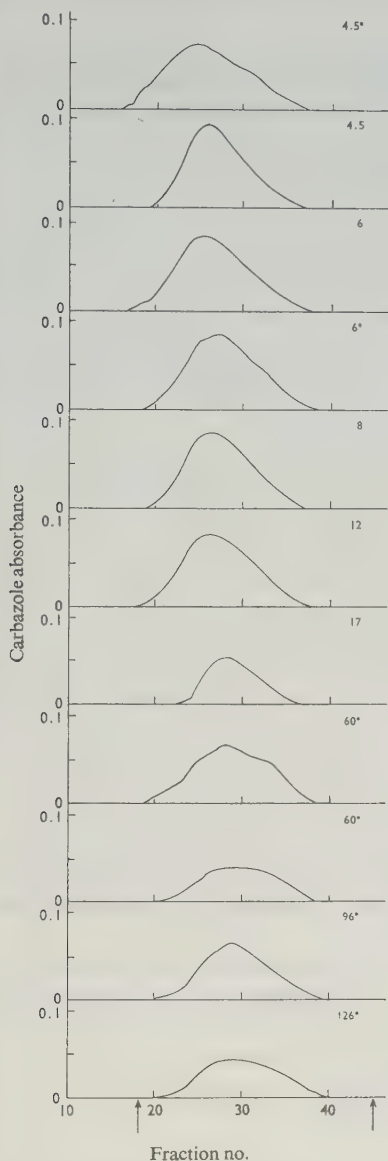


Fig. 3. Sepharose 2B column (1420mm×6mm) chromatograms of reduced and alkylated fraction A1 from the associative-density-gradient centrifugation from normal cartilage

The patterns represent the proteoglycan monomers. The void volume and the total volume are indicated by arrows. *Normal cartilage obtained from joint with degenerative changes.

size distribution of the chondroitin sulphate chains. The elution patterns indicate that there is only a very small decrease in the size of the chondroitin sulphate chains with increasing age of the cartilage (Fig. 4). Therefore it is likely that the decreasing size of the proteoglycan monomers can be attributed to a decreasing size of the proteoglycan protein core in the older cartilage, rather than to a decreasing size of the chondroitin sulphate side chains.

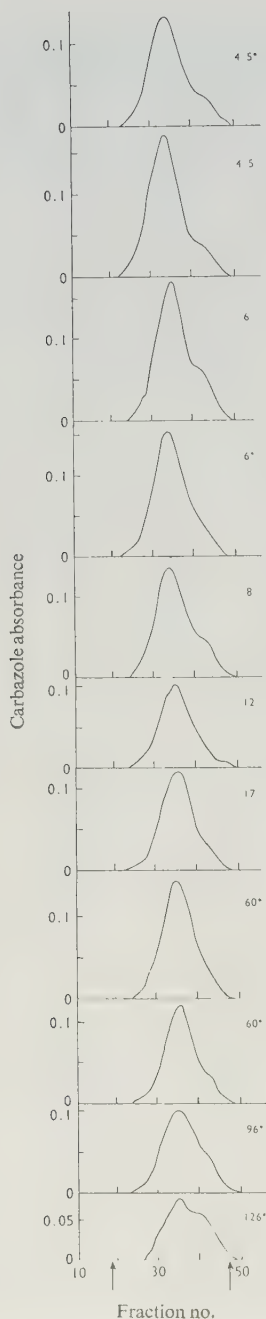
Degenerated cartilage in osteoarthritis

The large variations in proteoglycan composition in aging cartilage stresses the importance of using controls of comparable age when degeneration of cartilage is studied. Therefore all parameters of degenerated cartilage discussed below are compared with the same parameters of normal cartilage of the same age.

Composition. The total glycosaminoglycan content (expressed per wet weight of cartilage) was somewhat lower in degenerated than in normal cartilage of the same age (Table 1), although not statistically significant. It has been suggested (Matthews, 1953; Bollet *et al.*, 1963; Bollet & Nance, 1966; Mankin & Lipello, 1970) that such changes occur in osteoarthritis. The contents of total glycosaminoglycans when expressed per hydroxyproline contents, however, showed no difference when compared with normal cartilage, presumably owing to significantly lower ($P < 0.05$) contents of hydroxyproline per wet weight of the degenerated cartilage (Table 1). The lower hydroxyproline contents of degenerated cartilage are indicative of a higher water content in osteoarthritic cartilage. These data corroborate findings of others (Bollet & Nance, 1966; McDevitt & Muir, 1976; Mankin & Zarins-Trasher, 1975). Bollet & Nance (1966) and Mankin & Lipello (1970) also found that the collagen content related to tissue dry weight was the same in normal and degenerated cartilage. Similar results with increasing water content of the cartilage that contained smaller amounts of proteoglycans were observed in experiments by Lipshitz *et al.* (1976). They used enzymic treatment or extraction of cartilage with salt solutions to remove the proteoglycans.

Degenerated cartilage contained more keratan sulphate with increasing age (Table 2). Accordingly the keratan sulphate/chondroitin sulphate ratios increased, as did the keratan sulphate contents, both when related to wet weight and to hydroxyproline contents of cartilage. These changes are similar to, and statistically not significantly different from, the variations observed in the normal cartilage (Table 2).

Extraction of degenerated cartilage. The extraction yields of proteoglycans from the degenerated cartilage varied similarly to those observed for normal cartilage: decreasing yields with increasing age. There was, however, a significantly greater extraction



yield in the degenerated compared with normal cartilage ($P < 0.025$) (Table 1). Similar results have been obtained by others (McDevitt *et al.*, 1973; Brandt, 1974). Their procedures, however, were not designed to minimize effects of tissue proteinases and of other degradative enzymes. It is possible, then, that some degradation of the cartilage matrix may have obscured their results. Keratan sulphate content (expressed as percentage of total glycosaminoglycan content in the extracts) did not differ appreciably in degenerated compared with the normal cartilage (Table 2). The relative keratan sulphate content of the residues after extraction, however, was significantly higher ($P < 0.025$) in the degenerated cartilages. In contrast, the keratan sulphate content of the residues, when related to the hydroxyproline content, was not significantly different in the degenerated cartilage (Table 2).

Associative CsCl-density-gradient centrifugation. The proportion of the total proteoglycans recovered in fraction A1 from the degenerated cartilages had a tendency to decrease with increasing age of the cartilage (Table 3).

The composition of fraction A1 showed increasing protein and decreasing uronic acid contents with increasing age (correlation coefficients 0.65 and -0.45 respectively). Such changes were also noted for the normal cartilage (see above). The uronic acid/protein ratios of fraction A1 from the degenerated cartilages, however, were significantly higher ($P < 0.01$) than the ratios from the normal cartilage. Another difference was the significantly lower ($P < 0.005$) uronic acid contents of fraction A2 from the degenerated cartilage (Table 3). Since the proportion of the total uronic acid that was recovered in fraction A2 is the same for normal and degenerated cartilage, it is likely that the differences observed reflect the presence of a large proportion of non-proteoglycan material in the extracts of the degenerated cartilage.

Gel chromatography. Sepharose 2B chromatography of fraction A1 from the degenerated cartilage showed a smaller proportion of aggregated proteoglycans, and therefore more monomers, than did the elution patterns of proteoglycans from normal cartilage (Fig. 5). Such a difference could be due to absence of the hyaluronic acid-binding region from the proteoglycan monomers from degenerated cartilage (see below).

Fig. 4. Sephadex G-200 column (1465 mm $\times$ 6 mm) chromatograms of the papain-digested fraction A1 of the associative-density-gradient centrifugation from normal cartilage. The patterns represent the chondroitin sulphate side chains. The void volume and the total volume are indicated by arrows. *Normal cartilage obtained from joint with degenerative changes.

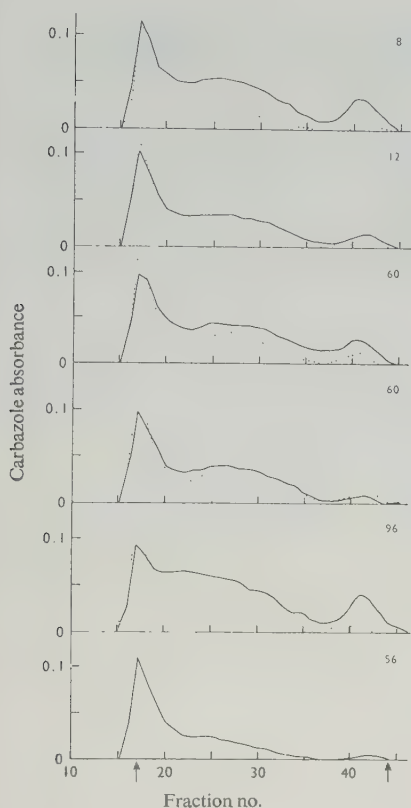


Fig. 5. Sephadex 2B column (1400mm×6mm) chromatograms of the fraction A1 from the associative density-gradient centrifugation from osteoarthritic cartilage (—) and from normal cartilage from the same individual (·····).

The size of the peaks is corrected so that the area under the void volume peak is constant. The void volume and the total volume are indicated by arrows.

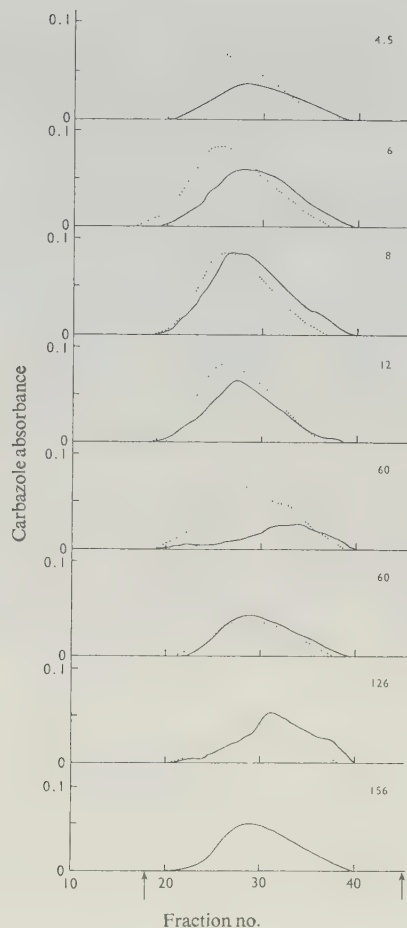


Fig. 6. Sephadex 2B column (1420mm×6mm) chromatograms of reduced and alkylated fraction A1 from the associative-density-gradient centrifugation from osteoarthritic cartilage (—) and from normal cartilage from the same individual (·····).

The patterns represent the proteoglycan monomers. The void volume and the total volume are indicated by arrows.

The proteoglycan monomers from the degenerated cartilages were smaller than those from normal cartilage, as shown by the more retarded peaks after Sephadex 2B chromatography (Fig. 6). Sephadex G-200 chromatography of papain digests of the proteoglycans was used to monitor the size of the chondroitin sulphate chains. The chromatograms (Fig. 7) indicated that the chondroitin sulphate chains in the proteoglycans (fraction A1) from the degenerated cartilage were not appreciably different from those of the control cartilages of the same age.

Owing to shortage of material only the samples from degenerated cartilage indicated in Figs. 5, 6 and 7 could be chromatographed.

The degenerated cartilage was macroscopically graded into four subclasses. There were, however, too few animals in each subgroup to allow for any

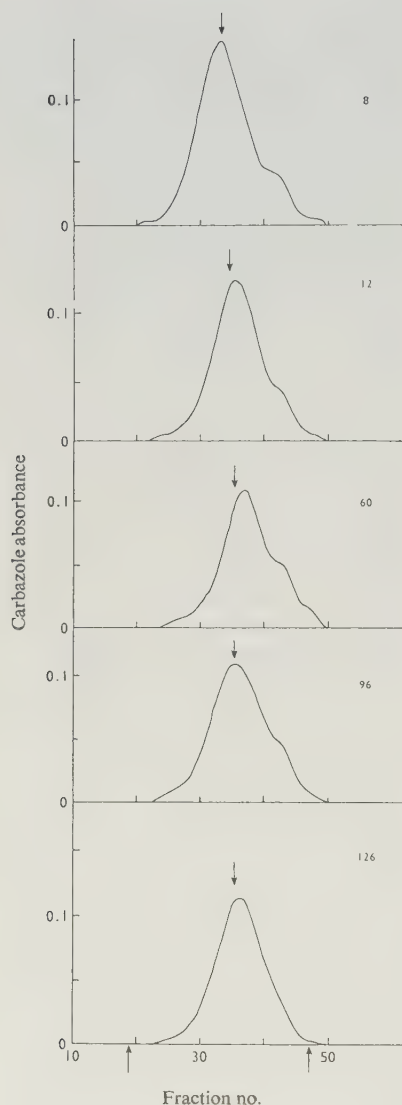


Fig. 7. Sephadex G-200 column (1465 mm $\times$ 6 mm) chromatograms of the papain-digested fraction A1 of the associative-density-gradient centrifugation from osteoarthritic cartilage

The patterns represent the chondroitin sulphate side chains. The arrows above the peaks indicate the elution peak of normal cartilage chondroitin sulphate chains (from Fig. 4). The void volume and the total volume are indicated by arrows.

attempt to statistically correlate the degree of degeneration to the structural changes of the proteoglycans.

General Discussion

The elastic properties of cartilage are determined by the three-dimensional organization and fixation of the charged groups (i.e. mainly the chondroitin sulphate chains) and therefore by the proteoglycan structure, rather than only by their quantity. Therefore the proteoglycan structure was studied; the investigation was, however, for technical reasons limited to the extractable proteoglycans. The asymmetric distribution of protein and glycosaminoglycan chains in cartilage proteoglycans is indicated in the current model of proteoglycan monomer structure shown in Fig. 1 (normal). The contents of protein, keratan sulphate and chondroitin sulphate in the proteoglycans can be utilized as markers, to discern whether the proteoglycans are altered in aging or in disease, as indicated in the protocol shown in Fig. 1. Minor and probably non-detectable differences are listed within parentheses. The size of the keratan sulphate chains will not be discussed because the normal chain size is not well established (Heinegård & Axelsson, 1977) and there is no documented technique for measuring the size of the keratan sulphate chains.

Articular-cartilage proteoglycans contain the keratan sulphate-rich region (D. Heinegård & I. Axelsson, unpublished work). The results of the various analyses show that with increasing age of the cartilage (1) the size of the proteoglycan monomers decreases, (2) the average size of the chondroitin sulphate chains does not change appreciably, (3) the chondroitin sulphate content, measured as contents of galactosamine and uronic acid, decreases, (4) the keratan sulphate content increases, (5) the protein content increases, (6) the ratio of aggregates to monomers, which can be used as an indication of the capability of the monomers to form aggregates or used as an indicator for the presence of hyaluronic acid-binding region, does not change and (7) the proportion of proteoglycans of lower buoyant density increases.

Thus the changing composition with age indicates that the proteoglycans undergo a gradual decrease in size. The change is probably due to a diminishing chondroitin sulphate-rich region, whereas the hyaluronic acid-binding region and the keratan sulphate-rich region remain comparatively constant. This change is consistent with the decreasing elasticity of the older cartilage since the chondroitin sulphate-rich region, which is most important for the elasticity of the cartilage, is decreasing in size. Results indicating similar changes in articular cartilage proteoglycan structure with age have been obtained by Strider *et al.* (1976).

Whether the changes observed are a result of an increased degradation of normally synthesized proteoglycans or a result of the biosynthesis of altered proteoglycans monomers is not answered by the present investigation, although some comments can be made. In the event of an increased degradation of proteoglycans, one would expect to find small-molecular-size chondroitin sulphate-rich peptides derived from the chondroitin sulphate-rich region. In the aging cartilage, no such fragments could be demonstrated. It is still possible, however, that such fragments are removed from the tissue very rapidly, and therefore not detectable.

All cartilage proteoglycan molecules, regardless of size, probably contain the keratan sulphate-rich region (Heinegård, 1977), whereas the chondroitin sulphate-rich region may vary. Therefore the keratan sulphate content of a cartilage is probably a better indication of the number of proteoglycan molecules (i.e. their molar concentration) in the tissue. Bearing this in mind it is noteworthy that the absolute keratan sulphate content of cartilage increases with age. Thus the molar concentration of proteoglycan monomers in the tissue probably increases with increasing age, contrary to what would be expected from the chondroitin sulphate contents. This observation may indicate either that the synthesis of proteoglycan monomers is increased or that their degradation and removal is decreased.

Only one animal was used in all but one age group and so no estimate could be made of variations within each group. It is unlikely, however, that the differences observed are due to variations between individuals. The trend with changes to a smaller proteoglycan with significantly higher keratan sulphate and protein contents with increasing age indirectly indicates that individual variations within each age group are of lesser importance.

All the parameters studied for the degenerated osteoarthritic cartilage showed similar variations with age as in the normal cartilage. However, a few differences were noted. The degenerated cartilage had a higher water content (wet weight divided by hydroxyproline contents), than normal cartilage of the same age. The extraction yield was significantly higher from osteoarthritic cartilage, and as indicated by the density gradient centrifugation more non-proteoglycan material was extracted. Such a result may imply a less tight network of the intercellular matrix, with less interactions between the various components. The reason may be an increased activity of proteinases, shown to be present in increased amounts in osteoarthritic cartilage by Ali & Evans (1973). Support for such a hypothesis derives from the other differences noted. The proteoglycans in fraction A1 from the degenerated cartilage had significantly higher uronic acid/protein ratios, although the molecules were smaller and contained

at least as much keratan sulphate. Further, gel chromatography of fraction A1 showed lower contents of proteoglycan aggregates in the degenerated tissues. The data thus indicate that an increased proportion of the proteoglycan did not contain the hyaluronic acid-binding region.

In conclusion, the data indicate that the proteoglycans from the degenerated cartilage were smaller than those from the same-age normal cartilage, and had partially lost their ability to bind to hyaluronic acid and form aggregates.

The changes of the proteoglycan structure in the degenerated cartilage were of different nature, compared with those occurring in normal aging articular-cartilage proteoglycans, regardless of whether the normal cartilage was obtained from a normal joint or from joints with areas with degeneration. It is possible, then, that the processes leading to degeneration are focal.

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Variations in the composition of bovine hip articular cartilage with distance from the articular surface

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Punch biopsies of bovine hip articular cartilage was sectioned according to depth and the proteoglycans were isolated. The mid-sections of the cartilage contained more proteoglycans than did either the superficial or the deepest portions of the cartilage. The most superficial 40 μm of the cartilage contained relatively more glucosaminoglycans compared with the remainder of the cartilage. The proteoglycans recovered from the surface 200 μm layer contained less chondroitin sulphate, were smaller and almost all of these molecules were able to interact with hyaluronic acid to form aggregates. From about 200 μm and down to 1040 μm from the surface, the proteoglycans became gradually somewhat smaller, probably owing to decreasing size of the chondroitin sulphate-rich region. The proportion of molecules that were able to interact with the hyaluronic acid was about 90% and remained constant with depth. The proteoglycans from the deepest layer near the cartilage–bone junction contained a large proportion of non-aggregating molecules, and the average size of the proteoglycans was somewhat larger. The alterations in proteoglycan structure observed with increasing depth of the articular cartilage beneath the surface layer (to 200 μm) are of the same nature as those observed with increasing age in full-thickness articular cartilage. The articular-cartilage proteoglycans were smaller and had much higher keratan sulphate and protein contents than did molecules isolated from bovine nasal or tracheal cartilage.

Cartilage has a high elasticity and resilience, largely because of the high content of proteoglycans in the tissue (Kempson *et al.*, 1971; Harris *et al.*, 1972; Scott, 1975; Kempson, 1975). The proteoglycans consist of a central protein core and a large number of charged and extended polysaccharide side chains. The proteoglycans therefore will occupy a very large domain. Several proteoglycans bind to one hyaluronic acid molecule, forming a very large proteoglycan aggregate. Part of the proteoglycan protein core is the hyaluronic acid-binding region, containing little or no polysaccharide. Another portion of the protein core, containing most of the keratan sulphate chains, constitutes the keratan sulphate region, probably interspaced between the hyaluronic acid-binding region and the part containing the chondroitin sulphate chains (Heinegård & Axelsson, 1977; Hascall & Heinegård, 1979).

The quantity of glycosaminoglycans is highest in the mid-portions of the cartilage and lower both towards the surface and towards the osteochondral junction (Stockwell & Scott, 1967; Maroudas *et al.*, 1969, 1973; Lipshitz *et al.*, 1976; Maroudas, 1979). It has also been shown that the glycosaminoglycan

glucosamine/galactosamine ratio increases with increasing depth in the cartilage (Stockwell & Scott, 1967). Such information, when related to the current model of proteoglycan structure, may indicate that the size of the proteoglycans is smaller in the deeper portions of the cartilage.

The present investigation was initiated to study the changes in articular-cartilage proteoglycan structure with distance from the articular surface.

Materials and methods

Chemicals

Aristar-grade HCl was obtained from BDH, Poole, Dorset, U.K. Papain and diphenylcarbamoyl chloride-treated trypsin were from Sigma Chemical Co., St. Louis, MO, U.S.A. Sepharose CL 2B, Sepharose 2B and Sephacryl S-200 were purchased from Pharmacia Fine Chemicals, Uppsala, Sweden. Tissue-Tec II (CM-cellulose) was obtained from Miles Laboratories, Naperville, IL, U.S.A. The other chemicals used were all of analytical grade. High-molecular-weight rooster comb hyaluronic acid (Healon) was a gift from Pharmacia. This prepara-

tion of hyaluronate chromatographs in the void volume of a Sepharose 2B column.

Tissue preparation

Femoral-head articular cartilages from three different 2-year-old bulls were used. Within 10 min after the death of the animals, their left hip joints were carefully opened and the femoral head was divided from the rest of the femur and removed. The synovial fluid was blotted off the articular surface with soft tissue paper. By using a specially made punch of diameter 20 mm, a cartilage-bone cylinder was prepared from the most weight-bearing area of the cartilage, just dorsal and lateral to ligamentum teres femoris. The specimen was immediately removed from the punch, frozen in liquid N_2 and transported to the laboratory immersed in liquid N_2 . The cartilage-bone cylinder was longitudinally divided into small pieces with a cross-sectional area of about 3 mm^2 . The surface of each of these pieces had only a minor curvature and was for practical purposes considered to have no curvature. Each piece was mounted to an aluminium cylinder with the aid of Tissue-Tec and fitted to a freeze-microtome. All operations were done with the tissue pieces frozen at -30°C . Serial sections of $20\text{ }\mu\text{m}$ thickness were prepared from the articular-cartilage surface to the cartilage-bone junction. Each cartilage piece yielded 62 sections. Cartilage from three animals was pooled separately into two sets of seven groups, with distances from the articular surface of 0–40, 40–240, 240–440, 440–640, 640–840, 840–1040 and 1040–1240 μm for animals A and C and 0–40, 40–120, 120–200, 200–280, 280–360, 360–440 and 440–1240 μm for animal B. Special care was taken to ensure that the cartilage specimens did not thaw during preparation and sectioning, to minimize degradation of the proteoglycans.

Histological investigation

A small cartilage-bone cylinder of diameter 2 mm was taken adjacent to the preparative punch biopsy, for histological investigation after staining with hematoxylin/eosin.

Preparation of proteoglycans

Proteoglycans were extracted from each pool of sectioned cartilage from animals A and B with 3 ml of 4 M-guanidinium chloride/0.05 M-sodium acetate, pH 5.8, containing the proteinase inhibitors EDTA (0.01 M), benzamidine hydrochloride (0.005 M) and 6-aminohexanoic acid (0.1 M) (Oegema *et al.*, 1975). Extraction was done for 24 h at 4°C on a shaker. Extracts and residues were separated by filtration through a nylon sieve, which was rinsed twice with 0.5 ml of the 4 M-guanidinium chloride solution used for extraction. The washings were pooled with the original extract and a sample of each pool was

removed for determination of extraction yield. The residues were stored frozen until processed further.

The extracts were dialysed against 10 vol. of 0.05 M-sodium acetate, pH 5.8, containing the proteinase inhibitors described above. The density of the dialysis residue was adjusted to 1.64 g/ml by addition of solid CsCl. The solutions (7 ml/tube) were then centrifuged at 4°C for 60 h in an MSE 65 ultracentrifuge in the $10 \times 10\text{ ml}$ titanium angle rotor at 35000 rev./min (85000 g, r_{av} 6.44 cm). After termination of the centrifugation, the tubes were frozen in an absolutely vertical position by carefully immersing them in liquid N_2 . While still frozen the tubes were cut with a saw in a special guide to give the bottom two-fifths (the A1 fraction) and the top three-fifths (the A2 fraction). The densities of the fractions were determined with a 300 μl constriction pipette as a pycnometer. The fractions were then dialysed against one change of 0.5 M-sodium acetate and then extensively against distilled water, and then freeze-dried.

Agarose/polyacrylamide-gel electrophoresis

Analytical agarose/polyacrylamide-gel electrophoresis of the reduced and alkylated A1 fractions from the different layers was carried out as described by McDewitt & Muir (1971). The gels were scanned by measuring the A_{520} .

Column chromatography

Analytical columns ($0.3\text{ cm} \times 140\text{ cm}$) of Sepharose CL 2B and Sephacryl S-200 were eluted with 0.5 M-sodium acetate, pH 7.0, at 4°C . Usually 150 μg of proteoglycan or digest thereof was applied. The uronic acid content of each fraction was determined by an automated version of the carbazole method (Heinegård, 1973). Alternatively about 25 μg of proteoglycan was chromatographed on a Sepharose 2B column ($0.5\text{ cm} \times 140\text{ cm}$), eluted with 0.25 M- Na_2SO_4 at 15°C with a constant-flow infusion pump. The effluent was continuously monitored for A_{206} with a Uvicord S monitor. At this wavelength the absorbance for protein is about 6 times that of glycosaminoglycans on a dry-weight basis.

The ratios of proteoglycan aggregates to monomers in the A1 fractions were determined after chromatography on Sepharose CL 2B. To ensure binding of all proteoglycan monomers capable of interaction, high-molecular-weight hyaluronic acid (1% of proteoglycan dry wt.) was added to the A1 fractions before chromatography (Hascall & Heinegård, 1979).

The size distribution of the proteoglycan monomers was determined by chromatography on Sepharose CL 2B and Sepharose 2B after reduction and alkylation of the A2 preparations (Heinegård, 1977).

Typically 25–150 µg of fraction A1 was dissolved in 150 µl of 4 M-guanidinium chloride/0.05 M-Tris/HCl/0.005 M-dithiothreitol, pH 7.6, and incubated at 37°C for 5 h. Iodoacetic acid (20 µl of a 0.1 M solution) was then added and the sample was incubated for 12 h at room temperature in the dark. Reduction and alkylation prevent aggregate formation by proteoglycan monomers (Heinegård, 1977).

The size distribution of the chondroitin sulphate chains was determined by chromatography on Sephacryl S-200 after digestion of 150 µg of fraction A1 with 1 µl of a suspension of crystalline papain (25 mg/ml) at 65°C for 3 h in 150 µl of 0.005 M-sodium phosphate buffer, pH 7.0, containing 0.005 M-EDTA and 0.005 M-cysteine hydrochloride.

Isolation and determination of glycosaminoglycans

Extraction yields were determined as follows. The residues and a known volume of the extracts from animal A were each dialysed against water and freeze-dried. The samples were then suspended in 500 µl of the buffer described above and digested with 2 µl of the suspension of crystalline papain at 65°C for 15 h. The released glycosaminoglycans were then isolated from the glycopeptide fraction by Ecteola-cellulose ion-exchange chromatography (Axelsson & Heinegård, 1975). Glycosaminoglycans were eluted with 2 M-HCl. Total tissue glycosaminoglycan content and distribution was measured after papain digestion of the sections from animal C with 15 µl of the papain suspension for 72 h at 65°C. Glycosaminoglycans were isolated as described above.

Chemical methods

Hexosamine contents of fractions were determined by using a Durrum automatic amino acid analyser after hydrolysis of samples in 4 M-HCl in sealed tubes under argon at 100°C for 10 h. Hydrolysis for determination of amino acids was with 6 M-HCl at 110°C for 24 h in sealed tubes under argon. The amino acids were then determined by using the amino acid analyser. Hydroxyproline

was determined as described by Stegemann & Stalder (1967).

Results and discussion

Morphology of tissue specimens

The articular cartilage showed no macroscopic sign of degeneration. The histology of the tissue biopsy showed three major regions. The region nearest to the surface extended from 0 to 40 µm and contained fibres and discoidal cells arranged parallel to the articular surface. The second region in the middle of the cartilage extended from 40 to 1040 µm and had an appearance typical for cartilage, with few cells and an abundant intercellular matrix. The region (200 µm) just above the cartilage–bone junction (1040–1240 µm from the cartilage surface) contained spheroidal cells and represents the proliferation area of the cartilage bone junction. The thickness of the articular cartilage measured in the microscope was 1250–1300 µm, in good agreement with the thickness obtained from the sum of the sections prepared for chemical investigation.

Distribution of glycosaminoglycans

Available data indicate that the collagen content of hip articular cartilage is constant at different depths of the cartilage (Lipshitz *et al.*, 1975; Maroudas, 1979). It was therefore assumed that the collagen content was constant and that the ratio of glycosaminoglycan hexosamine to hydroxyproline can be taken to indicate the proteoglycan content of the sections. One set of sections from animal C was digested with papain, and weighed samples from the digests were taken for hydroxyproline and glycosaminoglycan determinations. The higher content of glycosaminoglycans in the mid-sections compared with sections both from the surface and from the osteochondral junction (Table 1) corroborates previous data (Stockwell & Scott, 1967; Maroudas *et al.*, 1969; Lipshitz *et al.*, 1976; Maroudas, 1979). The content of glycosaminoglycan glucosamine, probably indicating keratan sulphate, varied only

Table 1. *Glycosaminoglycan contents of sections from articular cartilage from animal C*

Glycosaminoglycans are quantified as hexosamine of isolated glycosaminoglycans and collagen as hydroxyproline. Ratios are w/w.

Section	Glycosaminoglycan hexosamine hydroxyproline	Glycosaminoglycan galactosamine hydroxyproline	Glycosaminoglycan glucosamine galactosamine
0–40 µm	0.21	0.14	0.54
40–240 µm	0.34	0.25	0.35
240–440 µm	0.50	0.35	0.43
440–640 µm	0.50	0.36	0.39
640–840 µm	0.49	0.34	0.45
840–1040 µm	0.44	0.30	0.45
1040–1240 µm	0.27	0.18	0.47

Table 2. *Glucosamine/galactosamine ratio of glycosaminoglycan fractions of proteoglycan aggregate (A1) fraction, extract and extraction residue, and glycosaminoglycan extraction yields (animal A)*

Abbreviation: n.d., not determined.

Section	Glucosamine/galactosamine in glycosaminoglycan fraction			Extraction yield (% of total)	Glucosamine/protein in A1 fraction	Galactosamine/protein in A1 fraction
	Extract	Residue	A1 fraction			
0–40 μ m	0.81	0.74	n.d.	98.6	n.d.	n.d.
40–240 μ m	0.25	0.40	0.49	97.4	0.31	0.62
240–440 μ m	0.28	0.57	0.39	99.1	0.36	0.93
440–640 μ m	0.37	0.60	0.46	97.6	0.38	0.82
640–840 μ m	0.33	0.51	0.61	98.8	0.40	0.65
840–1040 μ m	0.41	0.48	0.66	96.3	0.36	0.55
1040–1240 μ m	0.32	0.55	0.48	96.5	0.43	0.89

Table 3. *Amino acid composition of the proteoglycans (A1 fractions) from the various tissue sections (animal A)*

Values are expressed as residues per 1000.

Amino acid	Section	...	40–240 μ m	240–440 μ m	440–640 μ m	640–840 μ m	840–1040 μ m	1040–1240 μ m
Asp			81	78	79	79	81	82
Thr			57	56	54	54	53	57
Ser			154	144	154	156	169	156
Glu			131	146	147	152	136	149
Pro			70	74	71	76	60	74
Gly			150	163	179	169	181	151
Ala			66	62	63	68	57	62
Cys			8	6	4	6	3	2
Val			50	47	44	42	48	51
Met			4	2	2	3	0	0
Ile			30	28	25	24	21	25
Leu			62	63	60	58	60	66
Tyr			19	24	21	21	19	21
Phe			28	30	27	26	22	24
His			21	16	15	15	20	15
Lys			35	35	29	27	40	34
Arg			34	27	26	23	30	31

little, although the surface layer contained relatively more.

Extraction of proteoglycans

Extraction yields (animal A) were much higher (96–99%; Table 2) than those reported previously from other types of cartilage (for references see Hascall & Heinegård, 1979). Possibly the smaller pieces of cartilage obtained by sectioning result in improved extraction.

Corroborating previous results (Inerot *et al.*, 1978), the residues from all tissue samples contained relatively more glucosaminoglycans than did the extracts. The ratio of glucosamine/galactosamine in the glycosaminoglycan fractions (Table 1) was very high in the 0–40 μ m section in both the extract and the residue. It is likely that this layer of the cartilage may contain relatively more keratan sulphate or heparan sulphate. Stockwell & Scott

(1967) showed that the surface layer of human articular cartilage had a higher content of glycosaminoglycan glucosamine, mainly in the sulphated-polysaccharide fraction. The second pool of slices, corresponding to 40–240 μ m depth, showed the lowest ratio. The relative glucosaminoglycan content, however, increased somewhat with further depth of the cartilage.

Composition of A1 fractions

Contents of hexosamines, amino acid and protein of A1 fractions (density 1.71 g/ml) from animal A were determined. The glucosamine/galactosamine ratios were higher than for the corresponding extracts, but still tended to increase with depth of the cartilage. The layer from the cartilage–bone junction, however, again had a lower ratio (Table 2). The relative content of glucosamine was much

higher than that observed from bovine nasal and tracheal cartilage (Hascall & Heinegård, 1974), indicating a higher relative content of keratan sulphate, corroborating data from other articular cartilages (Inerot *et al.*, 1978). The amino acid compositions of the A1 fractions were very similar (Table 3). Protein contents of the fractions were determined as the sum of the amino acids. The ratio of hexosamines to protein was calculated (Table 2). Unfortunately the amount of material recovered from the A1 fraction from the top section was not sufficient for further analysis. The keratan sulphate/protein ratio (determined as glucosamine/protein) increased somewhat with distance from the cartilage surface. The chondroitin sulphate/protein ratio (determined as galactosamine/protein) was comparatively low in the A1 fraction recovered from 40 to 240 μm . The A1 fraction from the section 240–440 μm had a higher relative chondroitin sulphate content than any of the other fractions. The chondroitin sulphate/protein ratio gradually decreased with increasing depth of the cartilage. The sample near the cartilage–bone junction had a higher relative content of galactosamine. In summary, the composition of the A1 fractions recovered from sections at 240–1040 μm showed that the relative contents of chondroitin sulphate decreased, the contents of protein increased and the contents of keratan sulphate remained relatively constant. The decreased chondroitin sulphate content was not due to decreasing size of the chains, as discussed below. The 40–240 μm -section A1 fraction contained the least chondroitin sulphate and probably also less keratan sulphate than the other fractions analysed.

Gel chromatography

The size of the proteoglycan monomers from the various layers from animal A was determined by Sepharose-2B chromatography of the reduced and alkylated A1 fractions. The uronic acid (glycosaminoglycan) tracings of the chromatograms (Fig. 1) showed that the size distribution of the proteoglycans in the top fraction analysed (40–240 μm) differed, in that these molecules were on the average considerably smaller than those of the deeper layers. The proteoglycan monomers recovered from the layer 240–440 μm were on average somewhat larger than any of the others. The tracings indicate that the average size of the proteoglycan monomers decreases (see Table 4), in the samples from 240 and down to 1040 μm from the cartilage surface. All the samples showed approximately the same polydispersity, except for the much less polydisperse sample from the surface layer (40–240 μm). The gel-chromatographic data are consistent with changes in the composition of the proteoglycan

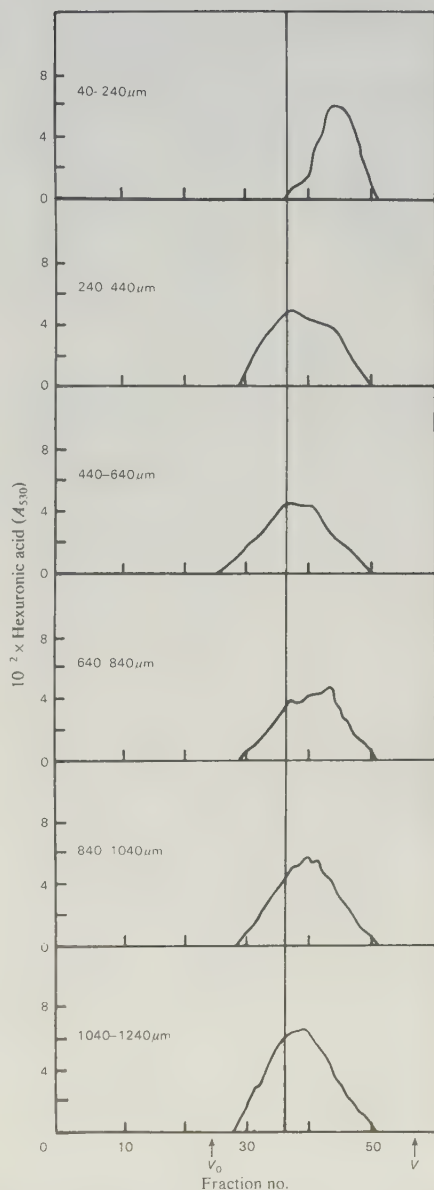


Fig. 1. Sepharose CL 2B chromatograms of proteoglycan monomers (reduced and alkylated A1 fractions from animal A)

The column (0.3 cm $\times$ 140 cm) was eluted with 0.5 M-sodium acetate, pH 7.0, in 300 μl fractions. The numbers indicate depth of cartilage layers from the surface. Abbreviations: V_0 , void volume; V_t , total column volume.

Table 4. Relative proportion of proteoglycans in fractions 27–36 and 37–51 in the chromatograms in Fig. 1

Distance from articular surface (μm)	Uronic acid (% of total in chromatogram)	
	Fraction 27–36	Fraction 37–51
40–240	0	100
240–440	32	68
440–640	39	61
640–840	21	79
840–1040	25	75
1040–1240	35	65

monomers discussed above. The top fraction analysed differs markedly, whereas the proteoglycans from the other sections show a tendency to a continuous change in the various parameters analysed. In order to determine the size of the proteoglycan monomers from the surface layer (0–40 μm) and to characterize the change in size of the proteoglycans in the uppermost 440 μm sections, the reduced and alkylated A1 fractions from the layers 0–40, 40–120, 120–200, 200–280, 280–360, 360–440 and 440–1240 μm (animal B) were separately chromatographed on Sepharose 2B. Because of the small amount of sample available, the procedure for analysis had to be altered. Instead of monitoring proteoglycans by uronic acid analysis, their A_{206} was continuously measured in column effluents, as discussed in the Materials and methods section. The chromatograms (Fig. 2) reveal that the proteoglycans in the uppermost 200 μm are distinctly smaller than those of the deeper layers and that the proteoglycans in the most superficial layer are of similar nature to those in the nearest layers.

It was considered that the changing size of the proteoglycan monomer may be due to changes in the size of the chondroitin sulphate chains. Therefore the size distribution of the chondroitin sulphate chains was determined by chromatography on Sephacryl S-200 of papain digests of the A1 fractions (animal A) (Fig. 3). The chromatograms, however, show that the size distribution of the chondroitin sulphate chains recovered from all the layers was very similar. Therefore other compositional parameters of the proteoglycan monomers were responsible for the variations observed.

The proteoglycan variability may be due to a changing proportion of aggregating and non-aggregating proteoglycans (Heinegård & Hascall, 1979). Therefore the A1 fractions (animal A) were chromatographed on Sepharose 2B after preincubation with hyaluronate to ensure that all proteoglycan monomers capable of interacting with hyaluronic acid would chromatograph in the void volume. The chromatograms (Fig. 4) indicate that

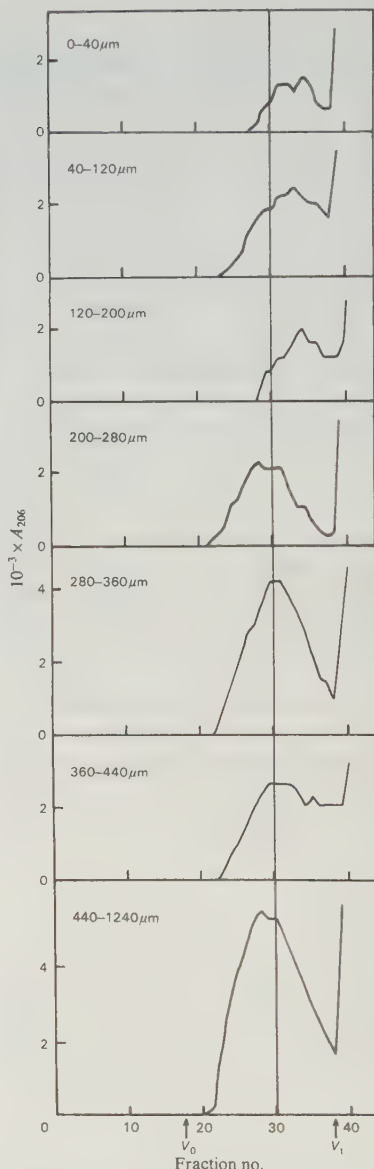


Fig. 2. Sepharose 2B chromatograms of proteoglycan monomers (reduced and alkylated A1 fractions from animal B)

The column (0.5 cm $\times$ 140 cm) was eluted with 0.25 M- Na_2SO_4 in 740 μl fractions. The A_{206} of the effluent was monitored with a LKB Uvicord S instrument. The numbers indicate depth of cartilage layers from the surface. The total-volume peak contains guanidinium chloride and dithiothreitol from reduction.

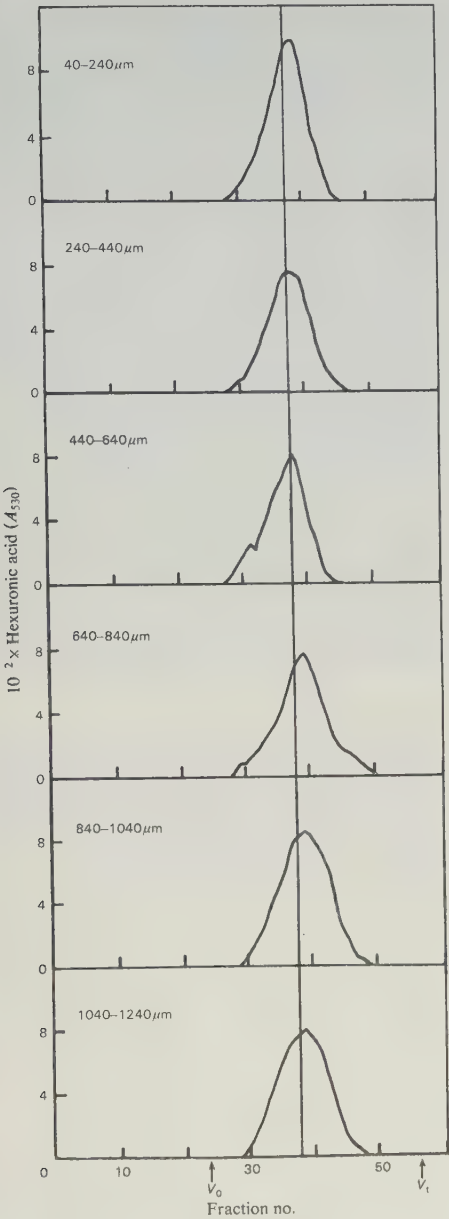


Fig. 3. *Sephacryl S-200* chromatograms of papain-digested A1 fractions from the layers indicated from animal A. The column (0.3 cm x 140 cm) was eluted with 0.5 M-sodium acetate, pH 7.0, in 300 μ l fractions. The numbers indicate depth of cartilage layers from the surface.

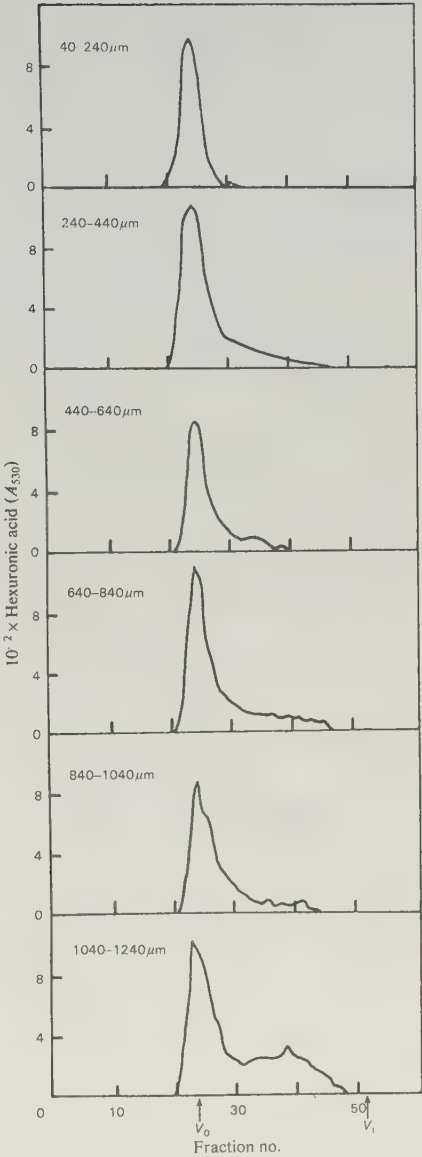


Fig. 4. *Sepharose CL 2B* chromatograms of A1 fractions from the different layers from animal A. Before chromatography the samples were incubated with 1% (w/w) of high-molecular-weight hyaluronic acid (Healon). The column (0.3 cm x 140 cm) was eluted with 0.5 M-sodium acetate, pH 7.0, in 300 μ l fractions. The numbers indicate depth of cartilage layers from the surface.

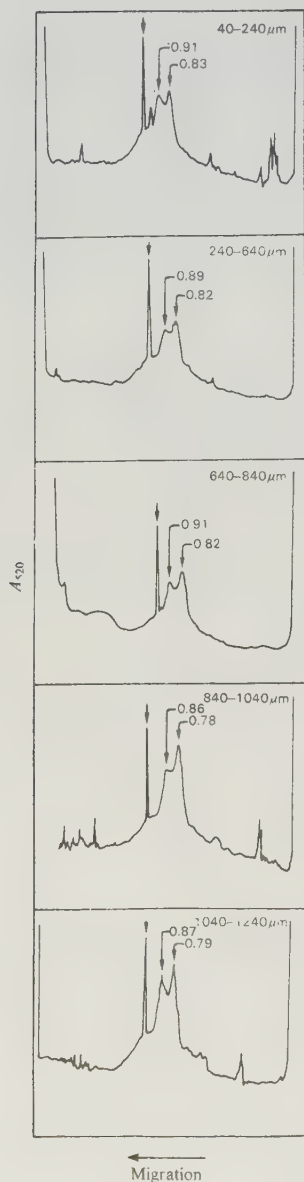


Fig. 5. Agarose/polyacrylamide-gel electrophoresis of reduced and alkylated A1 fractions from different layers.

The spectrophotometric scans at 520 nm are shown. The front marker (Bromophenol Blue) is indicated by an un-numbered arrow in each scan. The migration rate relative to that of Bromophenol Blue for the two or three components is indicated above the respective peaks. The numbers in μm indicate depth of layers from the surface.

the relative contents of aggregating proteoglycans did not change from the layers at 240–1040 μm . The top layer analysed (40–240 μm) contained only molecules capable of interacting with hyaluronic acid. The sample prepared from the proliferating zone near the cartilage–bone junction, on the other hand, contained a somewhat higher proportion of non-aggregating proteoglycans, possibly owing to changes in the cartilage matrix during cell proliferation and subsequent calcification.

Agarose/polyacrylamide-gel electrophoresis

On agarose/polyacrylamide-gel electrophoresis, cartilage proteoglycans migrate as two main components (Roughley & Mason, 1976). The scans of the agarose/polyacrylamide gel of the proteoglycans in the most superficial layer (40–240 μm) (Fig. 5) showed the two components. The scans of the preparations from the other sections showed only the two major bands. The proportion of these two bands changed so that the slower-migrating proteoglycan were somewhat more enriched with increasing depth of the cartilage (Fig. 5). The relative migration rate also decreased for both components in the deeper layers (840–1240 μm). These data indicate that there is more than one type of proteoglycan present in the articular cartilage. The smaller is enriched in the superficial layers.

General discussion

The data presented indicate that the proteoglycans isolated from the layers 0–200 μm differed from proteoglycans of deeper layers. They had a different composition, were of smaller size and all of them aggregated with hyaluronic acid. It should be pointed out, however, that there was an overlap in the chromatographic behaviour (Figs. 2 and 3). Therefore it is possible that the type of proteoglycans present in the most superficial layers (0–200 μm) may well have been present among the proteoglycans from the other layers, but then as one of several components. A small-molecular-size proteoglycan has previously been demonstrated in associative extracts of calf articular cartilage (Lipshitz *et al.*, 1976). The alterations in proteoglycan composition observed with increasing depth from 240 to 1040 μm indicate that the proteoglycan structure changes gradually from molecules containing a large chondroitin sulphate-rich region to molecules containing a smaller proportion of this region. It appears that in the intermediate sections of the cartilage the same large proportion of the molecules contained the hyaluronic acid-binding region and the keratan sulphate-rich region. The molecules found in the layer near the cartilage–bone junction had a different composition and contained a much larger proportion of non-aggregating proteo-

glycans. It is possible that some degradation of these molecules may have occurred *in vivo* and may be caused by proteinases released from the bone. The data presented indicate that the changes in proteoglycan structure with increasing age reported previously (Inerot *et al.*, 1978) are not due to losses of superficial layers of the articular cartilage. In comparison with proteoglycans isolated from nasal or tracheal cartilage of bulls of the same age, the articular-cartilage molecules are considerably smaller and have much higher content of keratan sulphate, corroborating previous data (for references see Muir, 1979). The molecules from the three tissues show essentially the same ability to interact with hyaluronic acid and form aggregates (Hascall & Heinegård, 1974; Heinegård & Hascall, 1979). These observations are consistent with a model where the hyaluronic acid-binding region and the keratan sulphate-rich region are constant, but the chondroitin sulphate-rich region varies (Heinegård, 1977).

It should be stressed that each set of data shown in the present paper were obtained from one punch biopsy from one animal. Therefore inter-individual variability does not obscure structural differences between layers. Other sets of chromatograms obtained with samples from another animal were essentially the same, indicating that the differences observed were not unique for one animal.

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Bovine Tracheal Cartilage Proteoglycans. Variations in Structure and Composition with Age

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Abstract

The variation with age in structure and composition of proteoglycans from bovine tracheal cartilage was studied from fetal life to 12 years of age. Cartilage content of proteoglycans as related to collagen was found to increase with age. The extractability of the proteoglycans decreases markedly with age. Extracted proteoglycans were characterized and change during growth and maturation as follows:

1. They contain fewer chondroitin sulfate chains. The chains are of constant size; but at higher ages they are more highly sulfated, primarily with 6-sulfate groups.
2. They contain an increasing number of constant size keratan sulfate chains, while the number of O-linked oligosaccharides (structurally related to the keratan sulfate linkage region to protein) decreases.
3. They become smaller, at least partially, as a result of an increasing proportion of a smaller size proteoglycan.
4. They have a higher protein content.

The changes can largely be attributed to an early shift (up to 22 months of age) from a predominating large chondroitin sulfate rich proteoglycan in the youngest cartilage to increasing proportions of a distinct, smaller keratan sulfate rich proteoglycan. During subsequent aging all molecular parameters as well as proportions of subpopulations of proteoglycans remain rather constant.

Key words: proteoglycan, aging, chondroitin sulfate; keratan sulfate, oligosaccharide.

Introduction

Hyaline cartilage contains few cells and an abundance of extracellular matrix, composed mainly of collagen and proteoglycans. The proteoglycan molecule consists of a central protein core to which a large number of highly negatively charged polysaccharide chains, chondroitin sulfate and keratan sulfate, are covalently attached. At one end, the protein core has a globular structure (Heinegård

and Hascall, 1974) to which no or very few polysaccharide chains are attached. This part of the protein core is capable of binding specifically to hyaluronic acid. Thus, several proteoglycans can bind to one hyaluronic acid molecule to form an aggregate. The main portion of the keratan sulfate chains and a few chondroitin sulfate chains are attached to a separate region of the protein core, the keratan sulfate rich region, located in close proximity to the hyaluronic acid binding region. The remaining 90 % of the chondroitin sulfate chains and some keratan sulfate chains are attached to the distal portion of the protein core, the chondroitin sulfate rich region (Heinegård and Axelsson, 1977). Recent data (Heinegård et al., 1981 b) indicate that cartilage actually contains two distinct populations of aggregating proteoglycans, those chondroitin sulfate rich, containing a prominent chondroitin sulfate rich region and those keratan sulfate rich, which in addition to a less prominent chondroitin sulfate rich region also contain a keratan sulfate rich region.

The composition of cartilage changes during fetal development, maturation and aging of the animal. The contents of chondroitin sulfate and water have been reported to decrease with age, while the contents of keratan sulfate and collagen increase (for references see Inerot and Heinegård, 1982). The composition of proteoglycans in aging articular cartilage was previously investigated by Campo and Tourtelotte (1967), Mutiah et al. (1973), Simunek and Muir (1972), Inerot et al. (1978), Roughley and White (1980) and Bayliss and Ali (1981). These studies show increased contents of keratan sulfate and protein. The size of the proteoglycan monomers decreases with age (Inerot et al., 1978), possibly due to a reduced size of the chondroitin sulfate rich region. In the present study, non-weight bearing hyaline tracheal cartilage was investigated with special reference to the variation of proteoglycan structure with age.

Materials and Methods

Tissue preparation

Bovine tracheal cartilage from male fetuses of 4.8 and 9 months gestational age and from steers 5, 11, 22, 41, 75, 78, 96, 126 and 141 months of age were obtained fresh from the local slaughterhouse. The age of all the animals will be indicated relative to birth, i.e. the fetuses were -4.5 and -0.3 months old, respectively. The cartilage was dissected free from soft tissues and perichondrium, frozen in liquid N₂ and ground under liquid N₂ in a Wiley mill. The powder was stored at -30 °C until extracted.

Extraction

Samples (200 mg) of cartilage powder were extracted for 24 h at 3 °C with 3.5 ml of 4 M guanidinium chloride/0.05 M sodium acetate pH 5.8, containing the protease inhibitors 10 mM EDTA, 100 mM 6-aminohexanoic acid and 5 mM benzamidinium chloride (Oegema et al., 1975). Extracts were centrifuged at 21,700 g (r_{av} 8.6 cm) for 20 min at 4 °C. The residues were twice resuspended in 0.5 ml of the extraction solvent and again centrifuged as above. The supernatants were added to the original extracts.

Purification of extracted proteoglycans

The concentration of guanidium chloride in the extracts was lowered to 0.4 M by dialysis against 9 volumes of 0.05 M sodium acetate, pH 5.8 (containing the protease inhibitors) for 24 h at 3 °C. The densities were adjusted to 1.61–1.62 g/ml by addition of solid cesium chloride and the solutions were centrifuged in an MSE 10 × 10 ml aluminium angle rotor at 34,000 rpm (83,000 g, r_{av} 6.46 cm) and 12 °C for 60 h (Hascall and Sajdera, 1969; Heinegård, 1972). Tubes were frozen vertically by immersion in liquid nitrogen and divided while still frozen (A1-fraction, bottom 40 %; A2-fraction, top 60 %).

In one set of experiments 250 mg of cartilage powder was extracted with 3.75 ml of 4 M guanidinium chloride containing the protease inhibitors. This extract was directly centrifuged in 4 M guanidinium chloride-cesium chloride with a starting density of 1.36 g/ml (Heinegård et al., 1981a). After centrifugation for 60 h in a Beckman Ti 70.1 rotor at 40,000 rpm (r_{av} 6.1 cm, 109,000 g) and 14 °C, the bottom 30 % of the gradient, which contained the proteoglycans, was recovered. After dialysis these samples (d.D1) were used for agarose-polyacrylamide composite gel electrophoresis.

Chemical methods

Protein contents were determined using the microprocedure of Lowry et al. (1951) and uronic acid contents by the procedure of Bitter and Muir (1962). Hydroxyproline (collagen) contents were determined as described by Stegeman and Stalder (1967) after hydrolysis of samples in 6 M HCl at 100 °C for 24 h.

The contents of galactosaminitol, glucosamine and galactosamine in fractions were determined by using an automatic amino acid analyzer after hydrolysis of samples in 4 M HCl under argon at 100 °C for 10 h. A modified buffer, 0.2 M sodium citrate, 0.15 M sodium chloride, pH 6.45, was used. Glucosamine, galactosamine and galactosaminitol elute in this order and are well separated.

Quantitation of glycosaminoglycans

Glycosaminoglycan contents of cartilage extracts and residues were determined as the sum of the glucosamine and the galactosamine contents of the glycosaminoglycans isolated after papain digestion and subsequent ion exchange chromatography on DEAE-cellulose (DE-32, Whatman, England) (Axelsson and Heinegård, 1975). Keratan sulfate contents of isolated proteoglycans were calculated from quantitations of glucosamine in the glycosaminoglycan fraction isolated after extensive chondroitinase and alkaline borohydride treatment of proteoglycans as is discussed below.

Extraction yields were determined from the hexosamine contents of the glycosaminoglycan fraction prepared from extracts and residues.

Characterization of keratan sulfate and oligosaccharides

Proteoglycans (A1-fractions) were digested with chondroitinase ABC (Heinegård and Axelsson, 1977), and disaccharides were removed by extensive dialysis against distilled water. Keratan sulfate chains were liberated from such core protein preparations by alkaline borohydride treatment at 5 mg/ml in 0.05 M sodium hydroxide, 1 M sodium borohydride, 50 h, 45 °C (Carlson, 1968). Using this procedure the

reducing terminal N-acetyl-galactosamine residues of keratan sulfate chains are quantitatively converted to N-acetyl-galactosaminitol (unpublished). The effect on the O-linked oligosaccharides, which have a similar linkage region (Lohmander et al., 1980) is assumed to be the same. The keratan sulfate chains were separated from oligosaccharides using chromatography on DEAE-celulose essentially as described by DeLuca et al. (1980). The oligosaccharides were eluted with 0.6 M pyridine acetate, pH 6.5, and keratan sulfate was eluted with 2 M HCl.

Quantitation of chondroitin 4- and 6-sulfate

Samples of proteoglycan A1-fractions were extensively digested with chondroitinase AC-II as described by Hascall et al. (1972). The unsaturated disaccharides formed, were separated on an HPLC weak anion exchange resin and quantified as described by Hjerpe et al. (1979) using detection by the carbazole procedure and UV absorption at 232 nm, respectively.

Gel chromatography

The proportion of aggregates and monomers in each A1-fraction was determined by gel chromatography on Sepharose 2B (Heinegård, 1972). The size distribution of proteoglycan monomers was determined by Sepharose 2B gel chromatography after reduction and alkylation of samples to abolish aggregation (Heinegård, 1977).

The size distribution of the chondroitin sulfate side chains was determined by gel chromatography (Wasteson, 1971) on Sephacryl S-200 after digestion of samples with papain as described elsewhere (Heinegård, 1977). Gel chromatography was performed on columns (140 or 160 $\times$ 0.6 cm) eluted with 0.5 M sodium acetate, pH 7.0, at a constant temperature of 3 °C. Fractions were analyzed for uronic acid content by an automated version (Heinegård, 1973) of the Bitter and Muir (1962) carbazole procedure.

Electrophoresis on agarose-polyacrylamide composite gels was performed essentially according to McDevitt and Muir (1971) using 3 mm thick slab gels. Samples (about 15 μ g in 20 μ l) were dissolved in the electrophoresis buffer and prior to electrophoresis incubated overnight with half their weights of hyaluronic acid oligosaccharide (HA-14) to abolish any interaction with high molecular weight hyaluronic acid.

Calculations

Linear regression correlation coefficients for the correlation of chemical data to age were calculated using the method of least mean squares. A students' t-test (Rao, 1965) was performed on each regression line to test the hypothesis that the line does not differ from a horizontal line.

Results and Discussion

Proteoglycan content and extractability

It is noteworthy that the glycosaminoglycan content of the cartilage, as related to collagen, Figure 1a, approximately doubles over the age period studied, although changes over the first 22 months are minor. The yields of proteoglycans

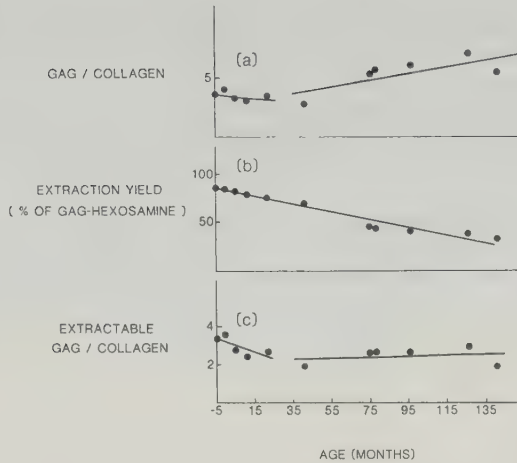


Fig. 1. Glycosaminoglycan (GAG) contents and extractability at various ages. Glycosaminoglycans were quantified as hexosamines (nmole) in the glycosaminoglycan fraction and collagen as hydroxyproline (mg). For details see text.

in 4 M guanidinium chloride extracts are similar to those previously described for other cartilages (Simunek and Muir, 1972; Inerot et al., 1978). The extraction yield ranged from almost 90 % in the fetal cartilage to 33 % in the very aged cartilage (Fig. 1b), with a significant decrease with age (correlation coefficient 0.83, $p < 0.001$). After the age of 22 months, the content of extractable proteoglycans remains rather constant, Figure 1c. In contrast the pool of nonextractable proteoglycans appears to increase with age. The increase in glycosaminoglycan content with age contrasts with the rather constant amount observed in aging dog hip articular cartilage (Inerot et al., 1978), indicating that weight bearing cartilage might change with age in a manner different from non-weight bearing cartilage.

Associative density gradient centrifugation

Proteoglycans, aggregates plus monomers, were purified by CsCl-density gradient centrifugation. The uronic acid content of the fractions was used to monitor for proteoglycans. The values obtained do, however, underestimate low molecular weight proteoglycans, which have low contents of chondroitin sulfate (Heinegård, 1977; Heinegård et al., 1981). The bulk of the proteoglycans was always recovered in the high density, A1-fraction, although this portion decreases somewhat from more than 90 % in cartilages from animals up to 41 months of age to about 80 % in older ages.

Composition of A1-fractions

In order to discern structural changes of extractable proteoglycans with age, the A1-fractions were further characterized. Proteoglycans contain a central pro-

tein core having attached side chains of chondroitin sulfate, keratan sulfate, O-linked oligosaccharides and N-linked oligosaccharides (Lohmander et al., 1980). In the following quantitative as well as some qualitative measures of these structural components will be discussed.

When interpreting a changing proteoglycan composition, one should bear in mind that cartilage contains two major populations of aggregating proteoglycans (Heinegård et al., 1981 b). The alterations in proteoglycan structure with aging may indeed be largely attributed to a changing proportion of these subpopulations as discussed below.

Changes of mainly keratan sulfate and chondroitin sulfate have often been expressed by an altered glucosamine to galactosamine ratio of the proteoglycans. The relative glucosamine content decreases during the period of growth and maturation, i.e. until 22 months of age and then remains rather constant, Figure 2. Indeed most structural features of the proteoglycans change during this early period of life, with only minor changes occurring later.

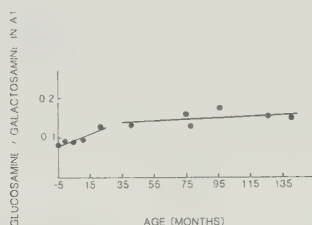


Fig. 2. Glucosamine to galactosamine ratios in A1 fractions of cartilage proteoglycans.

Chondroitin sulfate

The size of the chondroitin sulfate chains remains rather constant through life, as shown by their similar elution profiles from Sephacryl S-200, Figure 3. In contrast the number of chains per molecule as well as their composition changes during growth and maturation, Figure 4. Chondroitin sulfate contains alternating residues of glucuronic acid and N-acetyl galactosamine. The hexosamine may have a sulfate ester group at carbon 4 or 6 or be nonsulfated.

Since the size of the chondroitin sulfate chains remains constant, their number is proportional to the galactosamine as well as the uronic acid contents of chondroitin sulfate in the proteoglycan. The galactosamine content of keratan sulfate plus O-linked oligosaccharides in the proteoglycan represents a very minor proportion of the total galactosamine (cf. galactosaminitol over galactosamine in Figs. 4b, 6b) and furthermore remains rather constant, for instance, as related to protein (cf. Figs. 5a and 6a below). Therefore changes in galactosamine contents reflect changes in the number of chondroitin sulfate chains. The content of chondroitin sulfate side chains per molecule, then, decreases during growth and maturation, i.e. up to 22 months of age, as shown by the decreasing galactosamine to protein ratio (correlation coefficient -0.85), Figure 4a, as well as by a decreasing ratio

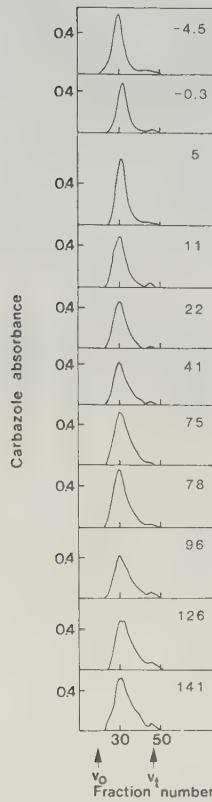


Fig. 3. Sephacryl S-200 (140 $\times$ 0.6 cm) chromatograms of A1-fractions digested with papain to demonstrate size distribution of chondroitin sulfate side chains. Elution was performed with 0.5 M sodium acetate, pH 7.0. Effluent fractions were analyzed for content of uronic acid using the carbazole procedure.

of galactosamine to number of keratan sulfate chains expressed as KS¹-galactosaminitol and further discussed below (correlation coefficient -0.83), Figure 4b. The galactosamine content of proteoglycans after 22 months of age remains rather constant as expressed by the ratios to protein as well as to KS-galactosaminitol, Figures 4b and 4c. Although the uronic acid content of the proteoglycans is not an exclusive measure of chondroitin sulfate, it decreases during growth and maturation (correlation coefficient $\log -0.98$ relative to protein and -0.82 relative to KS-galactosaminitol) only to remain relatively constant thereafter (data not shown).

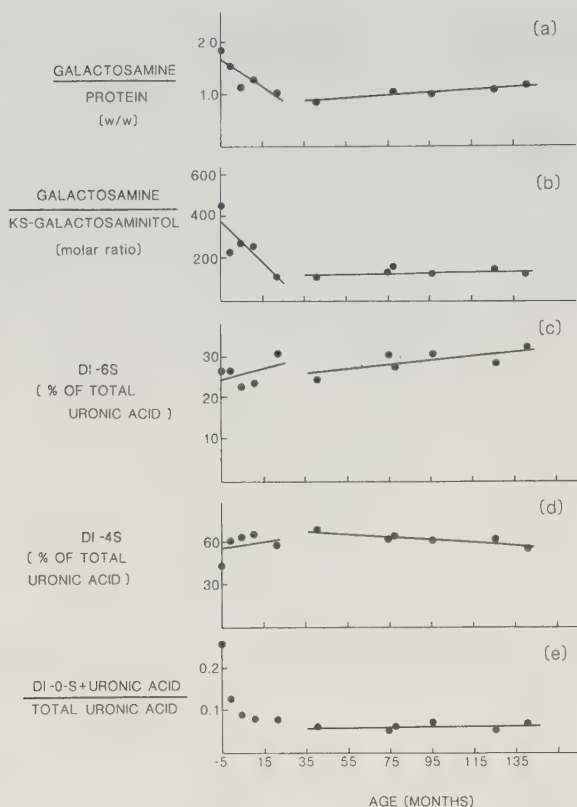


Fig. 4. Chondroitin sulfate in A1-fractions. Chondroitin sulfate was measured as contents of galactosamine. Disaccharides in chondroitinase digests of A1-fractions were quantified by HPLC. Keratan sulfate (KS) was quantified as described in the text.

The type of chondroitin sulfate in the proteoglycans changes. The relative proportion of Δ -di-6S in chondroitinase digests increases with age, Figure 4 c (correlation coefficient 0.69, $p < 0.02$ for the whole period studied) corroborating data from other investigations (Mathews and Glagov, 1966). The Δ -di-4S, on the other hand appears rather constant, Figure 4 d, during maturation, whereafter it shows a significant but rather small decrease (correlation coefficient -0.89 , $p < 0.05$). The separations of the chondroitinase digests show a retarded peak at the elution position of Δ -di-0S and/or free uronic acid. The amount of material in this component decreases mainly during fetal development from 26% of the total uronic acid (carbazole reaction) in the youngest cartilage, to about 5–7% in the older cartilage (Fig. 4 e).

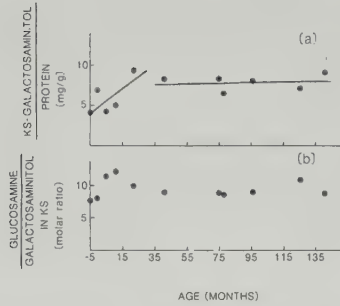


Fig. 5. Keratan sulfate in A1-fractions. The number of keratan sulfate chains was determined from the contents of galactosaminitol in glycosaminoglycans isolated from alkaline borohydride treated chondroitinase digests of A1-fractions. Glucosamine to galactosaminitol ratios of keratan sulfate is a measure of chain length.

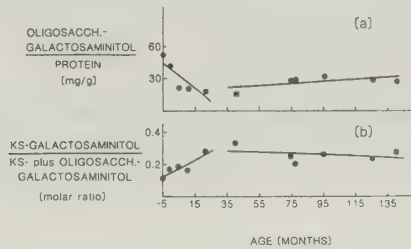


Fig. 6. Oligosaccharides in A1-fractions. Number of oligosaccharides and keratan sulfate (KS) chains were quantified by contents of galactosaminitol in the oligosaccharide and the keratan sulfate fraction, respectively, isolated by ion exchange chromatography of alkaline borohydride-treated chondroitinase digests of A1-fractions.

A complicating factor when considering the content of *A*-di-OS is the presence of hyaluronic acid in the cartilage. This hyaluronic acid, when bound in proteoglycans, is recovered in the A1-fraction. Previous studies by others (Bayliss and Ali, 1978; Venn, 1978; Elliot and Gardner, 1979), however, have shown that the contents of hyaluronic acid of articular cartilage increases with age. It is possible that the youngest fetal cartilage may have a somewhat higher content of hyaluronic acid. The overall agreement between uronic acid and galactosamine contents, however, indicates that the interference by hyaluronic acid is small.

Mathews and Glagov (1966), studying human costal cartilage, observed an increased sulfation of chondroitin sulfate during fetal development. The sulfation of the chondroitin sulfate appears to change with age to a higher substitution with 6-sulfate groups while the content of 4-sulfate groups changes much less. The

changes are much less prominent than those observed for human articular cartilage (Roughley and White, 1980), although in the latter case the contents of nonsulfated disaccharides were not given.

Keratan sulfate

Keratan sulfate contains repeat disaccharides of galactose and N-acetyl glucosamine having a sulfate group at carbon six. The chain is linked to protein via a specific oligosaccharide linkage region (Hopwood and Robinson, 1974; DeLuca et al., 1980). N-acetylgalactosamine is the reducing terminal sugar bound via an O-glycosidic bond to serine or threonine (Anderson et al., 1967). This bond can be cleaved by β -elimination using the reducing conditions described by Carlson (1968). This treatment quantitatively converts the linkage galactosamine to galactosaminitol (unpublished). Each liberated keratan sulfate chain, then contains one galactosaminitol residue. The isolated glycosaminoglycan fraction contains only galactosaminitol in keratan sulfate. When isolated after previous digestion with chondroitinase, which depolymerizes chondroitin sulfate as well as hyaluronic acid, all glucosamine in the glycosaminoglycan fraction is part of keratan sulfate chains. The galactosaminitol content of the glycosaminoglycan-fraction of alkaline treated A1-fractions, then, is a measure of the number of keratan sulfate chains, while the glucosamine to galactosaminitol ratio is a measure of the number of disaccharide repeat units of each chain, i. e. the chain length.

The number of keratan sulfate chains increases relative to chondroitin sulfate, Figure 4b, as well as to protein, Figure 5a (correlation coefficient 0.71) during the first 22 months of life, i. e. during growth and maturation. During subsequent aging the number of keratan sulfate chains appear rather constant.

The size of the keratan sulfate chains shows no distinct changes over the age period studied, Figure 5b. The values at young age are, however, considerably scattered.

Oligosaccharides

The N-linked oligosaccharides are bound to the hyaluronic acid binding region of the proteoglycan. They are of the complex type (for structural details see Nilsson et al., 1982). The O-linked oligosaccharides are structurally similar to the proposed linkage region of keratan sulfate and have been characterized in detail (Lohmander et al., 1980).

The nature of the oligosaccharides, whether O-linked or N-linked, was further studied. Only O-linked oligosaccharides contain a galactosamine residue, which forms the linkage to protein. This galactosamine, when reduced to galactosaminitol by alkaline borohydride treatment provides a measure of the O-linked oligosaccharides. Both types of oligosaccharides, on the other hand, contain glucosamine, which provides a measure of the total amount of oligosaccharide. The ratio of galactosaminitol to glucosamine in the oligosaccharide fraction shows very scattered values. No conclusion as to changes with age can be reached although the ratio appear somewhat higher during maturation, Table 1. More distinct measures of the number of O-linked oligosaccharides were obtained, Figure 6. During growth and maturation the number of O-linked oligosaccharide per protein decreases (correlation coefficient -0.85), Figure 6a, and remains rather constant during subsequent aging. The O-linked galactosamine residues are acceptors during

Table 1. Characteristics of oligosaccharides in A1-fractions (proteoglycans)

Age (months)	Glucosamine
	Galactosaminitol (molar ratio)
	Oligosaccharide fraction
-4.5 (fetal)	1.92
-0.3 (fetal)	1.79
5	2.06
11	3.54
22	3.39
41	2.68
75	2.92
78	2.16
96	2.13
126	2.09
141	2.56
Correlation coefficient	-0.07

biosynthesis for other monosaccharides to form oligosaccharides. These are hypothetically either terminated by sialic acid residues or elongated to keratan sulfate. The relative content of galactosaminitol in keratan sulfate over total galactosaminitol indeed increases during growth and maturation, Figure 6b (correlation coefficient 0.89), i. e. at the time when the number of keratan sulfate chains increases, Figure 5a, and the number of O-linked oligosaccharides decreases, Figure 6a. During subsequent aging the relative galactosaminitol content of oligosaccharides as well as keratan sulfate remains rather constant, Figure 6b.

The chemical data show that proteoglycan structure changes mainly during growth and maturation, i. e. up to 22 months of age. The content of chondroitin sulfate decreases while at the same time the keratan sulfate content increases. Possibly a larger number of the O-linked galactosamine residues are substituted with keratan sulfate rather than with oligosaccharides only. During subsequent aging the structure of the proteoglycans remains rather constant.

Gel chromatography of proteoglycans

Cartilage A1-preparations contain variable proportions of proteoglycan aggregates (Hascall and Heinegård, 1974; Heinegård and Hascall, 1979). Gel chromatography on Sepharose 2B is the most adequate procedure used to determine the proportion of aggregated to nonaggregated proteoglycans in the A1-fraction (Heinegård, 1972). Aggregates elute in the void volume, while monomers elute included. A1-fractions, however, contain a proportion of proteoglycan monomers capable of aggregating which are still not bound to hyaluronic acid and therefore elute included (Heinegård and Hascall, 1979). Such monomers will aggregate and shift to the void volume when incubated with high molecular weight hyaluronic

acid prior to chromatography. To ensure that all aggregating proteoglycans would chromatograph in the void volume, A1-fractions were incubated with hyaluronic acid (Healon, Pharmacia, Sweden) at a ratio of 50:1 (w/w). The proportion of nonaggregating proteoglycans was highest in the samples from the very youngest and the oldest animals, Figure 7, while lowest in samples from fetal to 4 years old animals. The distinct population of non-aggregating proteoglycans described

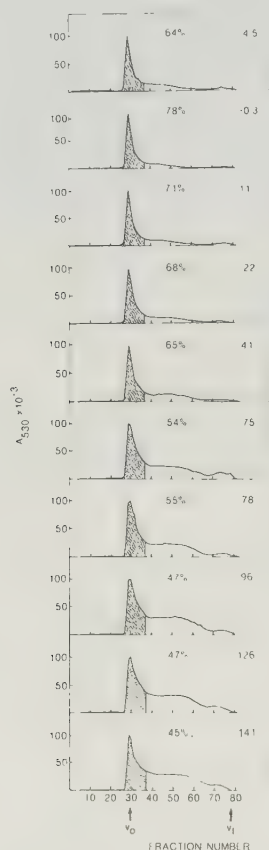


Fig. 7. Sepharose 2B (160 x 0.6 cm) chromatograms of the A1-fractions. Samples were incubated with 2% (w/w) of hyaluronic acid prior to chromatography. Elution was performed with 0.5 M sodium acetate, pH 7.0. Effluent fractions were analyzed for content of uronic acid using the carbazole procedure. The hatched areas represent the void volume component, i.e. aggregates, and the relative proportions of these components are indicated in the Figure. No sample was available from the 5 month old cartilage.

for bovine nasal cartilage have lower contents of glucosamine (Heinegård and Hascall, 1979), contrary to what is observed for proteoglycans from older individuals. The compositional alterations of proteoglycans observed with aging of the individual is therefore not explained by an increased proportion of such non-aggregating proteoglycans.

The size distributions of the proteoglycan monomers were determined by Sepharose 2B gel chromatography of the reduced and alkylated A1-fractions (Heinegård, 1977), Figure 8. The proteoglycan monomers eluted progressively

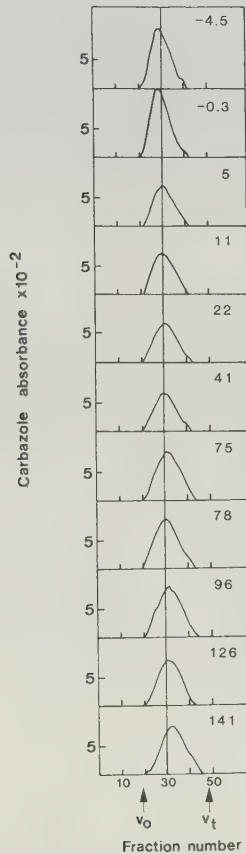


Fig. 8. Sepharose 2B (140 $\times$ 0.6 cm) chromatograms of the reduced and alkylated A1-fractions to demonstrate size distributions of proteoglycan monomers. Eluted fractions were analyzed for content of uronic acid using the carbazole procedure.

somewhat more retarded, i. e. are smaller, when isolated from older animals. The dimensions of the proteoglycan depend to a large extent on the size of the chondroitin sulfate rich region. The size of the chondroitin sulfate side chains remains constant through all age groups, Figure 2. The decreasing size of the proteoglycan monomers can therefore not be attributed to changes in the chondroitin sulfate chain size, but rather to a decreasing number of chondroitin sulfate side chains per proteoglycan in the older cartilage. Such a change in structure is consistent with an altered proportion of the subpopulations of aggregating proteoglycan as is discussed below.

Changes in proportions of proteoglycan subpopulations

The altered size and composition of proteoglycans with age may be explained either by structural changes in one type of proteoglycans or by an altered proportion of two or more proteoglycans of different composition. Recently, two populations of aggregating proteoglycans have been identified (Heinegård et al., 1981 b). The molecules in one population are smaller and contain less chondroitin sulfate while the content of keratan sulfate (glucosamine) and protein is higher. These molecules have a higher electrophoretic mobility in agarose-polyacrylamide composite gels (Heinegård et al., 1981 b). Samples from the d.D1-fractions were electrophoresed to reveal the proportions of the subpopulations, Figure 9. Prior to electro-

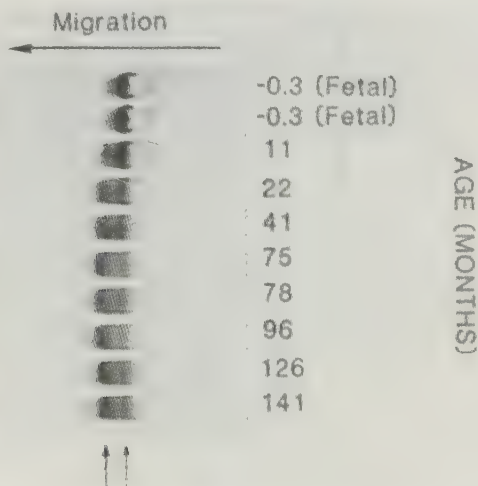


Fig. 9. Agarose-polyacrylamide composite gel electrophoresis of proteoglycans in d.D1-fractions. The arrows show the positions of reference chondroitin sulfate rich (1) and keratan sulfate rich (2) proteoglycans, respectively. Conditions are given in the text. No sample was available from the youngest fetal cartilage (-4.5 months) nor from the 5 month old cartilage. A sample from an additional fetal cartilage (39 weeks gestational age) was included in the electrophoresis.

phoresis the samples were treated with hyaluronic acid oligosaccharides to dissociate any aggregates. Two components can be observed in the cartilage from all adult animals, Figure 9. The component with highest mobility, corresponding to the keratan sulfate rich proteoglycan (Heinegård et al., 1981 b), becomes progressively more predominant with age. This component is very minor in the fetal cartilage samples, which contain a major component corresponding to the chondroitin sulfate rich proteoglycan (Heinegård et al., 1981 b). The relative content of the two populations of proteoglycans were determined from densitometric tracings of dried electrophoresis gels, Figure 10. Their proportions change markedly during growth and maturation, while being constant during subsequent aging. An additional minor component, Figure 9, with a lower electrophoretic mobility is observed in the fetal cartilage as well as in the youngest of the adult cartilages. Its nature is unknown. These data indicate that with age the proteoglycan pattern show a transition from a large, chondroitin sulfate rich, protein poor and keratan sulfate poor proteoglycan to a predominant, smaller keratan sulfate and protein rich proteoglycan. Immunochemical data show that the two types of proteoglycans are antigenically different and therefore probably represent different gene products (Heinegård et al., 1981 b).

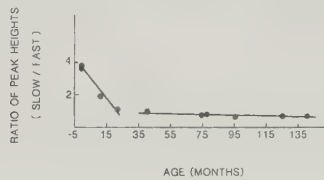


Fig. 10. Relative proportions of chondroitin sulfate rich and keratan sulfate rich proteoglycans in proteoglycans (d.D1-fractions) at various ages. Peak heights were measured in scans of the electrophoretograms shown in Fig. 9.

The present data corroborate and extend those presented by Triphaus et al. (1980). They showed that human xiphoid cartilage contains two pools of newly synthesized proteoglycans: a predominating chondroitin sulfate rich proteoglycan in young cartilage and a predominating keratan sulfate rich proteoglycan in old cartilage.

Discussion

The observed changes in the composition of extractable proteoglycans with age, support and extend previous observations (Inerot et al., 1978; Roughley and White, 1980; Roughley et al., 1981; Bayliss et al., 1981), i. e. aging cartilage contains smaller proteoglycans with more protein and keratan sulfate and contains a higher proportion of the proteoglycan with high electrophoretic mobility. The data can be taken to indicate that the major change in the proteoglycan composition results from a shift from one population of proteoglycans being predominant

in fetal life to an increased proportion of another population of proteoglycans during growth and maturation. Whether the composition within the two subpopulations also change remains to be answered. Tracheal cartilage proteoglycans, however, change less markedly after maturation compared with the articular cartilage proteoglycans previously studied. It is possible that tracheal cartilage, not being weight bearing and having a more homogeneous structure, is less prone to the effects of aging.

Acknowledgements

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Footnotes

¹ Abbreviations used: KS-keratan sulfate; /1-di-4S and /1-di-6S-disaccharides with 4-O-sulfate and 6-O-sulfate ester groups respectively produced by chondroitinase digestion of chondroitin sulfate.

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STRUCTURE AND COMPOSITION OF ARTICULAR CARTILAGE PROTEOGLYCANS
IN EXPERIMENTAL OSTEOARTHRISIS

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ABSTRACT

Degenerative hip joint disease was induced in dogs by extraarticular surgery that created a condition which mimics hip dysplasia. Osteotomy of the ramus of the iliac and ischiatic bone on the left side was followed by inward rotation of the osteotomized part of the pelvis. Cartilage degeneration followed from a decreased acetabular coverage of the femoral head.

For comparison a degenerative knee joint disease was induced in other dogs by transection of the anterior cruciate ligament of the knee.

The femoral head articular cartilage showed macroscopic signs of degeneration within a month. No macroscopic changes of synovitis were present. Chemical analysis of cartilage samples showed loss of proteoglycans even before any macroscopical signs of articular cartilage degeneration appeared. Guanidine hydrochloride extracts of the cartilage contained proteoglycan fragments which could be separated by density gradient centrifugation in cesium chloride. The data indicate that in degenerative joint disease, i.e. in osteoarthritis, proteoglycans are fragmented by proteolytic cleavage. The fragments are then lost from the cartilage.

The changes in proteoglycans observed in the experimental knee joint osteoarthritis were of a slightly different nature, possibly indicating that both degradation and repair took place in the knee articular cartilage. This may follow from different surgical procedures, only the one used for the hip joint being extraarticular, or from the different anatomy and physiology of the hip joint and the knee joint.

INTRODUCTION

Articular cartilage consists mainly of water (60-80%) held in an abundant extracellular matrix. The cells, chondrocytes, are not evenly distributed throughout the tissue, i.e. the cell density is higher in the surface layer of the cartilage. The matrix is composed of collagen (70% of the cartilage dry weight), proteoglycans (20-30% of dry wt) and small amounts of other tissue proteins (for refs see 15). Collagen forms fibers that are responsible for the tensile strength of the cartilage while the proteoglycans are the main factor determining the elasticity (compressive stiffness). Articular cartilage proteoglycans are macromolecules with molecular weights of about $1.1-1.6 \times 10^6$ daltons (25,42). They contain a central protein core, to which a large number of highly negatively charged glycosaminoglycan chains are covalently attached. Two types of glycosaminoglycans have been identified, i.e. chondroitin sulfate containing galactosamine and keratan sulfate, containing glucosamine. The proteoglycans are bound to hyaluronic acid by non covalent specific bonds, thereby creating large proteoglycan aggregates with molecular weights of the order of $50-100 \times 10^6$ daltons. A region, containing no glycosaminoglycan chains, at one end of the proteoglycan core protein (the hyaluronic acid-binding region), represents the interaction site.

The main feature of osteoarthritis is degeneration of the articular cartilage. The earliest morphologically identifiable sign of degeneration is a focal lesion of the weight-bearing surface of the cartilage. It is usually observed macroscopically as loss of glossiness and histologically as fibrillation of the surface of the cartilage. In some cases, fibrillation may be preceded by loss of stainability and metachromasia of the matrix of the surface layer of the articular cartilage. With increasing severity, clefts and surface erosion are found and eventually the articular cartilage is lost and eburnation of the subchondral bone follows. Osteoarthritis is common both in humans and in domestic animals. About 20% of cases of osteoarthritis in man are considered to be caused by a preexisting condition leading to incongruence of the articulating surfaces (8). In about 80% of the cases, such a preexisting condition cannot be identified, and the lesion is therefore called primary osteoarthritis. This disease is insidious in onset with symptoms and signs appearing rather late in the development. By the time the symptoms are severe enough to get the patient to seek care, the changes in the cartilage are usually well advanced. To disclose the early changes in osteoarthritis, it has therefore been necessary to develop animal models in which the early changes can be studied (for references, see 32). The most widely used experimental procedure is induction of knee joint instability by transection of the anterior cruciate ligament of the knee in dogs (22,33,34).

A new model for the induction of osteoarthritis was developed by Olsson *et al.* (32). By osteotomy and inward rotation of the osteotomized part of the pelvis, a hip dysplasia-like condition is induced. The change in load-bearing of the femoral head triggers a cartilage degeneration without concomitant acute synovitis, a complication in models in which an intraarticular approach is used.

In osteoarthrotic cartilage, the compressive stiffness and tensile strength of the cartilage is reduced (18,19). This is consistent with loss of proteoglycan and an injury to the collagen network. Chemical studies of osteoarthrotic cartilage, in support, have shown marked loss of proteoglycans (4,5,6,23, 24,39,43). Furthermore the structure of remaining proteoglycans is altered (for refs see 15). McDevitt *et al.* (26) and McDevitt & Muir (25) reported an early increase in the ratios of galactosamine/glucosamine and of uronic acid/protein in the proteoglycans isolated from knee articular cartilage taken from dogs, in which an experimental osteoarthritis had been induced by transection of the anterior cruciate ligament. In a study of osteoarthritis caused by naturally occurring hip dysplasia in dogs (16) the structure of proteoglycans from the degenerated hip articular cartilage was shown to be altered. The isolated proteoglycan monomer had a smaller size and a higher uronic acid/protein ratio and the proportion of aggregates with hyaluronic acid was reduced. It has been suggested that degradation of the proteoglycans is an important factor in the development of osteoarthritis (14,21,44). In support Ali and Evans (1), Shoji & Granda (39) and Sapolsky *et al.* (37,38) reported increased activity of cathepsins and neutral proteases and concomitant loss of uronic acid in osteoarthrotic cartilage. The reported changes of proteoglycan structure are consistent with partial proteolytic scission of the molecule.

One problem in studies of the degenerative process in experimentally induced osteoarthritis is the inflammatory process caused by the intraarticular surgery. The inflammation may in itself interfere with normal turnover of cartilage macromolecules (9,41). The aim of the present investigation was to study changes in proteoglycans in early osteoarthritis with no inflammatory response. For comparison, a commonly used model for knee joint osteoarthritis, in which synovitis is a complication, was also studied.

MATERIALS AND METHODS

Extensive analyses were carried out on cartilage samples (taken 15-59 days after surgery) from 4 one year old litter mate greyhounds of racing lineage. In addition samples primarily used for histology taken 119 and 153 days after surgery from two German wire haired pointers, were also included (series 2, Table I). From another series of greyhounds the samples removed were too small for complete analysis (series 3, Table I). Only some analytical data from these dogs are therefore included. All dogs were operated on with pelvic osteotomy as described elsewhere (32). At the time of sacrifice cartilage was excised from areas of the femoral heads that showed focal degeneration, identified by discoloration, loss of normal glossiness or erosion of the cartilage. When no such sign of degeneration was seen, cartilage was taken from a semilunar area just dorsal to ligamentum teres. This area is the main weight-bearing portion of the cartilage, where also most of the changes are usually seen in more advanced cases. Normal cartilage from the non-operated side was obtained from corresponding sites.

Three mongrel dogs of medium size, one year of age, were operated on with transection of the anterior cruciate ligament of their left knee. After 27, 107 and 134 days the dogs were sacrificed. Normal and degenerated cartilage was excised from corresponding sites on the area of the tibial plateau not covered by the menisci.

Isolated pieces of cartilage were immediately frozen in liquid nitrogen, stored at -80°C until pulverized in liquid nitrogen as has been described previously (16). Extraction was performed as described elsewhere (10) using 4 M guanidine-HCl solutions containing protease inhibitors (31).

The proteoglycan aggregates (A1-fraction) were isolated by CsCl gradient centrifugation in 0.4 M guanidine-HCl, pH 5.8 starting density 1.62 g/ml as described earlier (16). The proportions of aggregate/monomer in the A1-fractions were determined by chromatography on Sepharose 2B. To obtain size distribution of all the proteoglycan monomers by chromatography on Sepharose 2B, the protein in the hyaluronic acid binding region of the proteoglycan monomer was unfolded by reduction and alkylation to prevent the proteoglycan monomers from forming aggregates with hyaluronic acid. Size distribution of the chondroitin sulphate side chains was obtained by chromatography on Sephadex G 200 of papain digested A1-fractions. Hexosamines were quantified after hydrolysis of samples in 4 M HCl for 10 hrs at 100°C in sealed tubes under argon. A Durrum automatic amino acid analyzer was used as described previously (17). Glucosamine and galactosamine contents in glycosaminoglycans were determined after chromatography of

papain digests of proteoglycans on DEAE-cellulose (Whatman DE32) to purify the glycosaminoglycans as described elsewhere (2). It was assumed that glucosamine provided a measure of keratan sulfate (and hyaluronic acid), galactosamine a measure of chondroitin sulfate and their sum a measure of total glycosaminoglycans or proteoglycans. Hyaluronic acid was quantified after treatment of A1-fractions (12 µg/ml) in 0.1 M sodium hydroxide for 40 hrs at 20°C. A radioligand method using ¹²⁵I-labeled hyaluronic acid binding region was used (Wieslander and Heinegård, unpublished). Hydroxyproline contents in extracts and residues were determined according to Stegeman & Stalder (40). Uronic acid was determined according to Bitter & Muir (3) or by an automated version of this method (11). Protein was determined using the microprocedure of Lowry et al. (20).

RESULTS AND DISCUSSION

A. Hip model osteoarthritis

The articular cartilage from the joint which was operated on showed degenerative lesions in the greyhounds that were killed at postoperative day 19, 26, 36 and 59 and in the German wire haired pointers killed at 119 and 153 days after the operation. Severe degeneration, i.e. erosion, was observed in the dogs that were killed 36, 59, 119 and 153 days after the operation. Two dogs, killed 10 and 15 days after surgery showed no macroscopical signs of degeneration. The macroscopical findings indicate that the induced secondary osteoarthritis is progressive and gradual in development (Table I).

Decreasing contents of glycosaminoglycans in degenerating articular cartilage have been a frequent finding in osteoarthritis (6,16,21,26). In the present investigation, at 15 days after operation, the articular cartilage from the operated hip had a considerably lower content of glycosaminoglycans compared with the control side, Fig. 1. The cartilage samples taken later in the postoperative period all contained less glycosaminoglycans in the cartilage of the hip operated on. The changes seemed to be more marked with time. It appears that a very early sign of degenerative joint disease is loss of glycosaminoglycans, i.e. proteoglycans, from the affected cartilage. In analogy available data show that loss of metachromasia is an early event in osteoarthritis (for ref. see 15).

Studies of structural variations of the remaining proteoglycans in the articular cartilage could provide information on mechanisms of degradation and removal of the molecules from the tissue. Proteoglycans were therefore extracted using 4 M guanidine-hydrochloride. The amounts of extracted proteoglycans as well as proteoglycans in the extraction residue were lower from the hip which was operated on, even shortly after the operation,

Fig. 1b and 1c. It is possible, however, that extractable proteoglycans are lost from the tissue to somewhat greater extent compared with those in the residue, Fig. 1b and 1c.

Extracted proteoglycans were purified by density gradient centrifugation under associative conditions to yield the aggregate A1-fraction. Major portions of protein, keratan sulfate and chondroitin sulfate are enriched in three regions of cartilage proteoglycans, the hyaluronic acid binding region, the keratan sulfate rich region and the chondroitin sulfate rich region, respectively (12). Therefore changes in the relative proportions of these structures may be interpreted in terms of removal of specific regions by degradation of the proteoglycans. Analysis of isolated proteoglycans (A1-fractions) showed increased relative contents of chondroitin sulfate (measured as uronic acid in glycosaminoglycans) compared with protein contents, Fig. 2. The ratio increased more markedly with increasing time after the operation. The changes in contents of uronic acid truly represent altered contents of chondroitin sulfate, since the amounts of hyaluronic acid in the preparations are the same (discussed below). The keratan sulfate (glucosamine) contents of the purified proteoglycans (A1) decreased already a short time after the operation, Fig. 3a. McDevitt et al. (27) similarly observed decreased glucosamine contents of A1-fractions isolated from articular cartilage of dogs after the anterior cruciate ligament had been transected. The changes observed in the glucosamine contents can be attributed to an altered content of keratan sulfate, since separate quantitation of hyaluronic acid using a specific radioligand method showed essentially equal amounts of hyaluronic acid in all samples (Table II). The compositional data indicate that the proteoglycans purified from the articular cartilage of the side operated on are enriched in the chondroitin sulfate rich region and have decreased contents of keratan sulfate rich region. In addition lower protein contents indicate that the proteoglycans contain less of the hyaluronic acid binding region, which constitutes a substantial portion of the protein in the molecule. The data can be taken to indicate a fragmentation of the proteoglycans such that the high buoyant density chondroitin sulfate rich region was recovered in the bottom, A1-fraction, while protein and perhaps keratan sulfate rich fragments were recovered in the upper portions of the gradient used for purification. Support for this hypothesis was obtained from analysis of the extract, Fig. 3b. No significant difference was observed between relative keratan sulfate (glucosamine in glycosaminoglycans) contents of the extracts from the articular cartilage taken from the two sides. Since the recovery of keratan sulfate in the A1-fractions was lower from the cartilage of the hip operated on, it appears that some of the keratan sulfate was lost to the top fraction of the gradient. The data, therefore, strongly indicate that the extract from the articular

cartilage of the side operated on contained fragmented proteoglycans and that the major proportion of the material in A1 represented structures of the chondroitin sulfate rich region. The third series of greyhounds showed similar changes in the composition of the femoral head cartilage from the hip operated on. The uronic acid to protein ratio was increased although not until 36 days after operation, Table III. Furthermore, the relative content of glucosamine was higher in proteoglycans isolated from the side operated on, Table III.

Chromatography of the A1-fractions on Sepharose 2B showed that already after 19 days the proportion of aggregated proteoglycans from the hip operated on was greatly reduced and remained so for all the later times, Fig. 4. In the case of the German wire haired pointers, additional A1-samples were chromatographed after the addition of hyaluronate (2% w/w). The elution profile was identical to that when no hyaluronate had been added. It therefore appears that the low proportion of aggregates is not an effect of loss of hyaluronate from the A1-preparations. The low aggregatability and the lower relative protein contents, Fig. 2, of the A1-fraction indicate that the proteoglycans from the hip operated on do not contain a hyaluronic acid binding region. Chromatography on Sepharose 2B of reduced and alkylated A1-fractions, Fig. 5, showed that at 19 days and later in the post-operative period the proteoglycans from the side operated on eluted more retarded and were considerably smaller, indicating degradation. This is not an effect of smaller chondroitin sulfate chains, since their size was the same in all samples, Fig. 6.

Taken together the data provide evidence for an early scission of the proteoglycan protein core in degenerating articular cartilage. As a result a large fragment of the chondroitin sulfate rich region is formed. It has a high buoyant density and is recovered in the A1-fraction. Other fragments containing keratan sulfate and possibly rich in protein have low buoyant density. They are therefore lost in the top fraction of the gradient. It is possible that such fragments remain bound to the hyaluronic acid as has previously been described for aggregates fragmented in vitro to yield chondroitin sulfate rich peptides and hyaluronic acid with bound protein and keratan sulfate rich structures (12).

B. Knee model osteoarthritis

The same approach in terms of compositional analysis was used in studies of the cartilage of three dogs that were operated on with transection of the anterior cruciate ligament to induce knee joint osteoarthritis. This part of the study served as a control for the model of hip osteoarthritis described in this paper.

The cartilage from the operated knee of all dogs showed degenerative lesions (from roughness of surface to erosion)(see Table I). The menisci were severely torn and the cartilage lesions were located to the area not covered by the menisci. The cartilage content of glycosaminoglycans decreased with time after the operation, Fig. 7a, in agreement with observations in other studies (6,16,21,26), as well as analogous to the results obtained with the hip joint model.

In analogy to the results obtained using the hip joint model extractable as well as non-extractable proteoglycans were lost from the tissue, Fig. 7b and c. Further analysis showed that with time the composition of extracted proteoglycans became altered indicating degradation of the molecules, Fig. 8 and 9. Similar results have previously been published by McDevitt et al. (26,28). The changes in the structure of extractable proteoglycans, however, occurred at a much later time compared with those observed with the hip articular cartilage proteoglycans, although they were of the same nature.

The results from studies of proteoglycans aggregates were somewhat variable, Fig. 10. There were however no major differences in the proportion of aggregated proteoglycans from the joints operated on compared with the control joints. McDevitt et al. (28) also observed that proteoglycans isolated from degenerating knee articular cartilage did not show impaired aggregatability. The average size of the proteoglycan monomers from the side operated on were rather similar compared with those from the control side, Fig. 11. Control chromatograms on Sephadex G-200 of papain digested proteoglycans showed that the average size of the chondroitin sulfate side chains was the same in all knee cartilage samples (data not shown).

GENERAL DISCUSSION

A problem in studying changes in the chemical composition of a tissue undergoing a process, is to find points of reference. We have chosen to use contents of hydroxyproline, assuming that during the early stages of degenerative joint disease the amount of collagen in the cartilage remains rather constant. It should be noted that the water content of the tissue appears to increase, particularly at later times (data not shown). Two different types of induced degenerative joint disease have been studied. In hip joint osteoarthritis induced by osteotomy of the pelvis to mimic hip dysplasia, indeed the molecular changes are very similar to those observed in secondary osteoarthritis due to hip dysplasia (16). Early molecular changes include substantial loss of proteoglycans from the tissue. Structural changes of remaining, extractable proteoglycans indicate fragmentation of the molecules into substructures containing different regions of

the proteoglycan. It is likely that this cleavage is proteolytic in nature. In hip osteoarthritis induced by this extraarticular procedure there is apparently no inflammatory component. This type of experimental osteoarthritis therefore probably represents a truly degenerative joint disease in its early stages, where the degeneration is induced by an altered load-bearing. A schematic interpretation of the observed changes in molecular structure of the proteoglycans during developing hip osteoarthritis is shown in Fig. 12.

The degenerations induced in knee articular cartilage by sectioning of the anterior cruciate ligament represents a more complex situation. First of all, this joint has an entirely different anatomy and the surfaces of the articular cartilages are only in direct contact in a small area. The menisci modify load and impact. Secondly, the intraarticular surgery gives rise to synovitis. The inflammatory response may release factors like catabolin, which may trigger chondrocytes to degrade their surrounding cartilage (9,41). It appears that the changes in molecular composition of the knee cartilage, although occurring much later than the changes in the hip joint, are similar in nature. In contrast to proteoglycans isolated from the hip joint, those from the knee joint did not show reduced proportion of aggregates or a decreased size, supporting previously published data obtained with the knee joint model (26,28).

While the compositional data obtained with knee cartilage indicate fragmentation of the proteoglycans, the data on function, i.e. aggregating capacity and size distribution do not indicate that degraded molecules are retained in the tissue. One possible explanation is that in the knee articular cartilage the repair process is more active. This repair process could replace lost proteoglycans containing higher proportions of keratan sulfate and protein, with proteoglycans being rich in chondroitin sulfate. Indeed there are two subpopulations of aggregating proteoglycans in cartilage, one poor in keratan sulfate and one enriched (13). In support Sandy et al. (36) observed that in a response to loss of proteoglycans, rabbit cartilage cultured in vitro responded with a several-fold increase of the synthesis of chondroitin sulfate rich proteoglycans.

In conclusion it appears that the presented hip model for inducing osteoarthritis, causes a degenerative disease with early scission of proteoglycans and their subsequent loss from the tissue. Repair processes seem to be negligible since only very small amounts of 50 mCi ^{35}S -sulfate administered intravenously were incorporated in newly synthesized hip articular cartilage proteoglycans, i.e. in the order of 15 to 30 dpm per μg uronic acid in isolated proteoglycans. The induced disease is similar in nature to that of spontaneously occurring secondary osteoarthro-

sis in dogs with hip dysplasia (16). The present data furthermore show that the changes occur early in the development of experimental osteoarthritis.

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Table I. Listing of dogs included in the study and appearance of the articular cartilage studied. Hip operated dogs

Series	Breed of dog	Side	Days after operation	Macroscopic appearance
1	greyhound	non-op	15	normal
		op	15	normal
1	greyhound	non-op	19	normal
		op	19	loss of glossiness
1	greyhound	non-op	26	normal
		op	26	rough surface, yellow discoloration
1	greyhound	non-op	59	normal
		op	59	erosion
3	greyhound	non-op	10	normal
		op	10	normal
3	greyhound	non-op	19	normal
		op	19	slight loss of glossiness
3	greyhound	non-op	36	normal
		op	36	erosion
2	German Wirehaired pointer	non-op	119	normal
		op	119	deep erosion
2	German Wirehaired pointer	non-op	153	normal
		op	153	erosion
Knee operated dogs				
	Mongrel	non-op	27	normal
	Mongrel	op	27	rough surface
	Mongrel	non-op	107	normal
	Mongrel	op	107	erosion
	Mongrel	non-op	134	normal
	Mongrel	op	134	erosion

Table II. Hyaluronic acid contents of A1-fractions from the hip articular cartilage of the dogs operated on. Values expressed as ratio to uronic acid.

Days after operation	hyaluronic acid uronic acid (w/w)
15 non. op.	0.028
op.	0.026
19 non. op.	0.049
op.	0.041
26 non. op.	0.020
op.	0.023
59 non. op.	0.036
op.	0.040
119 non. op.	not determined
op.	0.062
153 non. op.	0.122
op.	0.082

Table III. Composition of A1 fractions of hip articular cartilage from the third series of dogs operated on (hip operated greyhounds).

Time after operation (days)	Side	Glucosamine total Hexosamine (w/w)	Uronic acid Protein (w/w)
10	non-op	0.154	0.825
	op	0.140	0.815
19	non-op	0.194	0.795
	op	0.170	0.780
36	non-op	0.198	0.745
	op	0.164	0.905

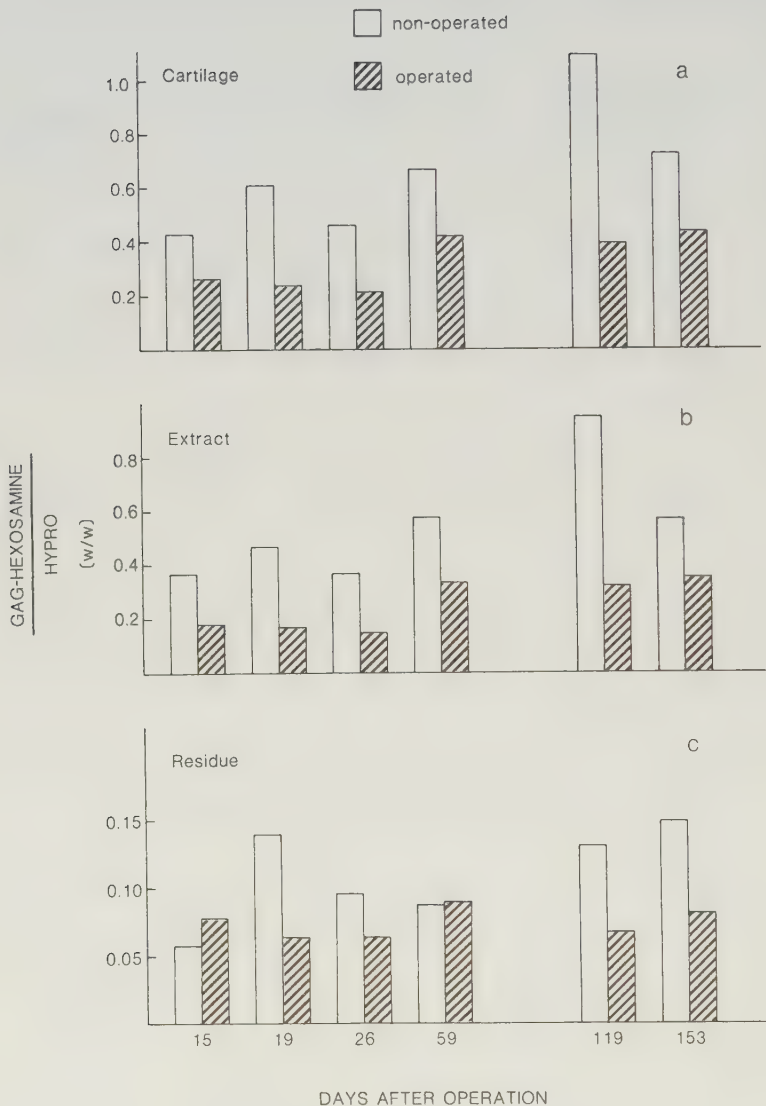


Figure 1. Proteoglycan content at various times after hip operation. Proteoglycans in the cartilage (a), extracted with 4M guanidinium chloride (b) and residue after extraction (c). Proteoglycans quantified as glycosaminoglycan-hexosamine over hydroxyproline (w/w).

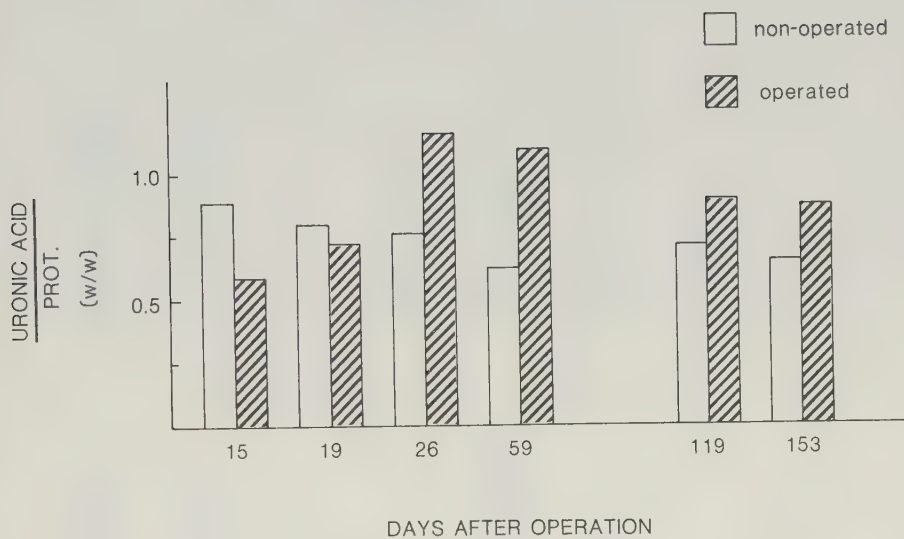


Figure 2. Uronic acid to protein ratio (w/w) of isolated proteoglycans A1-fractions at various times after hip operation.

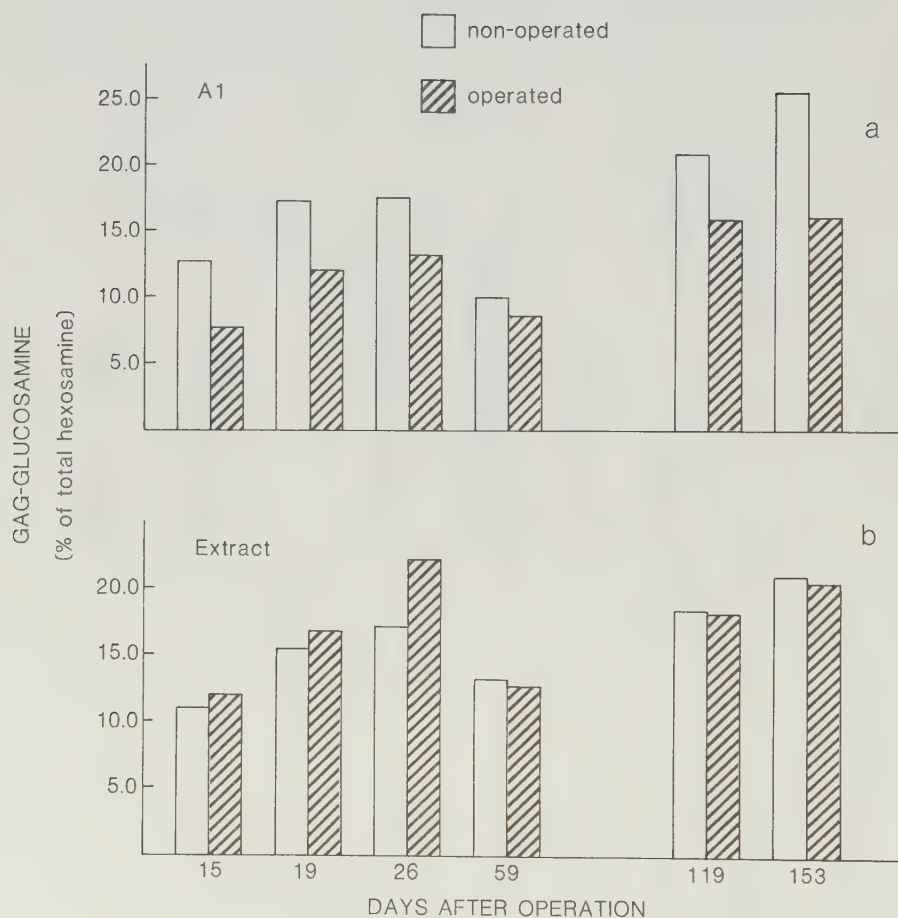


Figure 3. Ratios of glycosaminoglycan-glucosamine (keratan sulfate) to total glycosaminoglycans in isolated proteoglycans (A1) (a) and in guanidinium chloride extracts (b) of femoral head articular cartilage after hip operation.

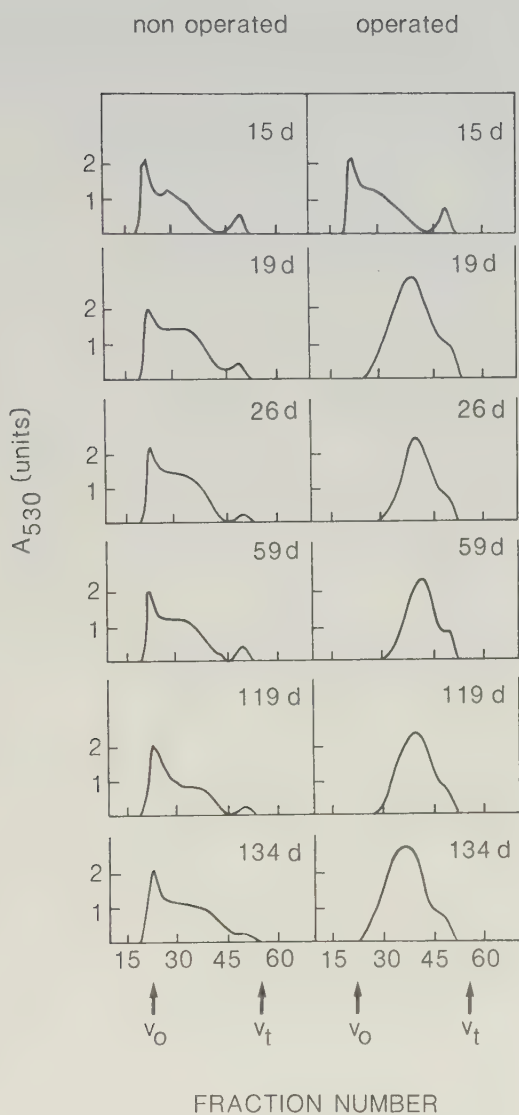


Figure 4. Chromatography on Sepharose 2B of the A1-fractions from the dogs operated on with a pelvic osteotomy. Operated and non-operated control sides shown to the right and left, respectively. The time intervals after surgery are from top to bottom: 15, 19, 59, 119 and 153 days. The absorbance (A_{530}) of the carbazole reaction is given as arbitrary units.

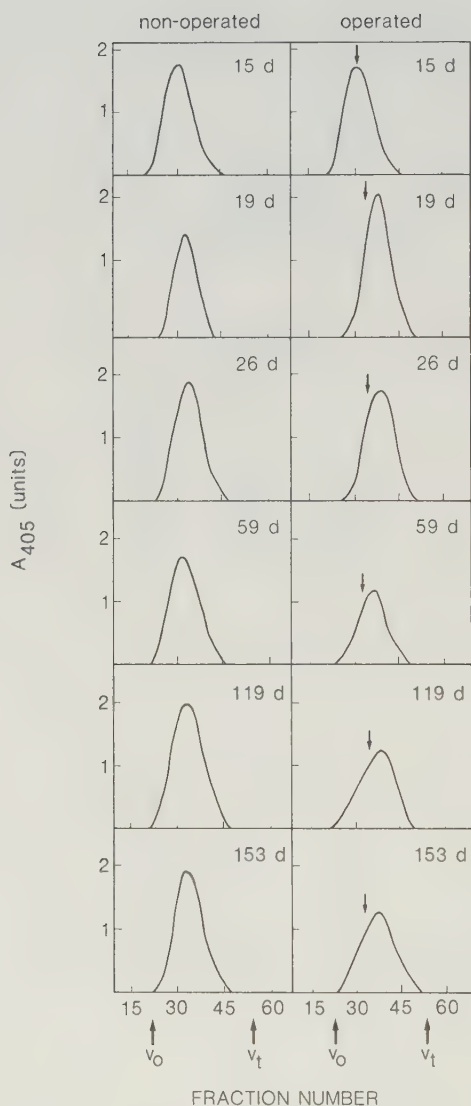


Figure 5. Chromatography on Sepharoe 2B of the reduced and alkylated A1-fractions from the dogs operated on with a pelvic osteotomy. Operated and non-operated control sides, shown to the right and left, respectively. The time intervals after surgery are from top to bottom: 15, 19, 26, 29, 119 and 153 days. The arrows in the chromatograms from the side operated on show the position of the peak of the control side. The absorbance (A_{530}) of the carbazole reaction is given as arbitrary units.

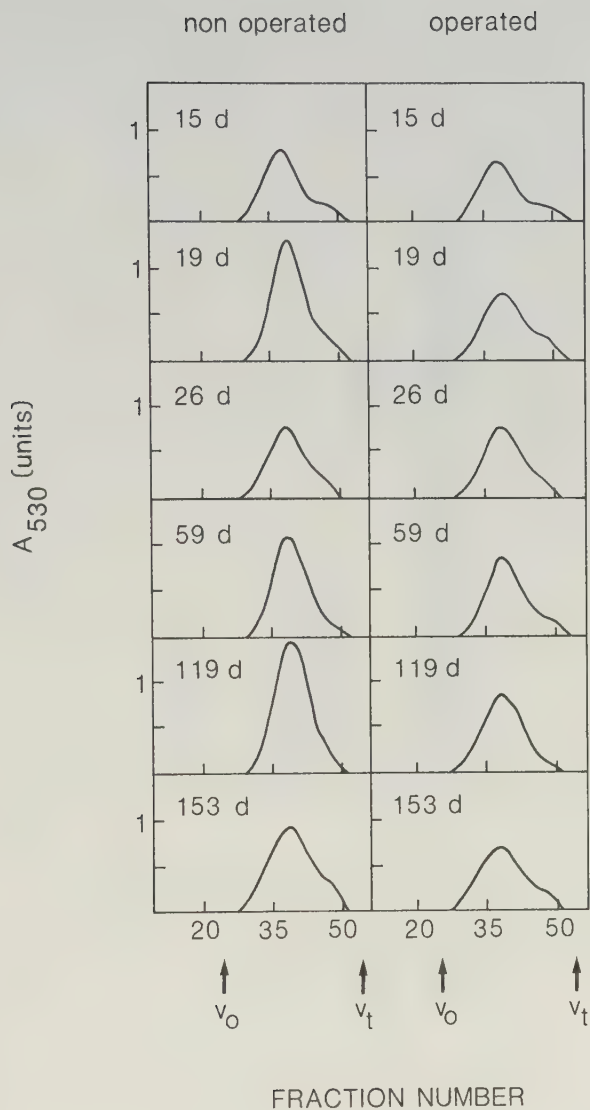


Figure 6. Chromatography on Sephadex G 200 of the papain digested A1-fractions from the dogs operated on with a pelvic osteotomy. Operated and non-operated sides shown to the right and left, respectively. The time intervals after surgery are from top to bottom: 15, 19, 59, 119 and 153 days. The absorbance (A_{530}) of the carbazole reaction is given as arbitrary units.

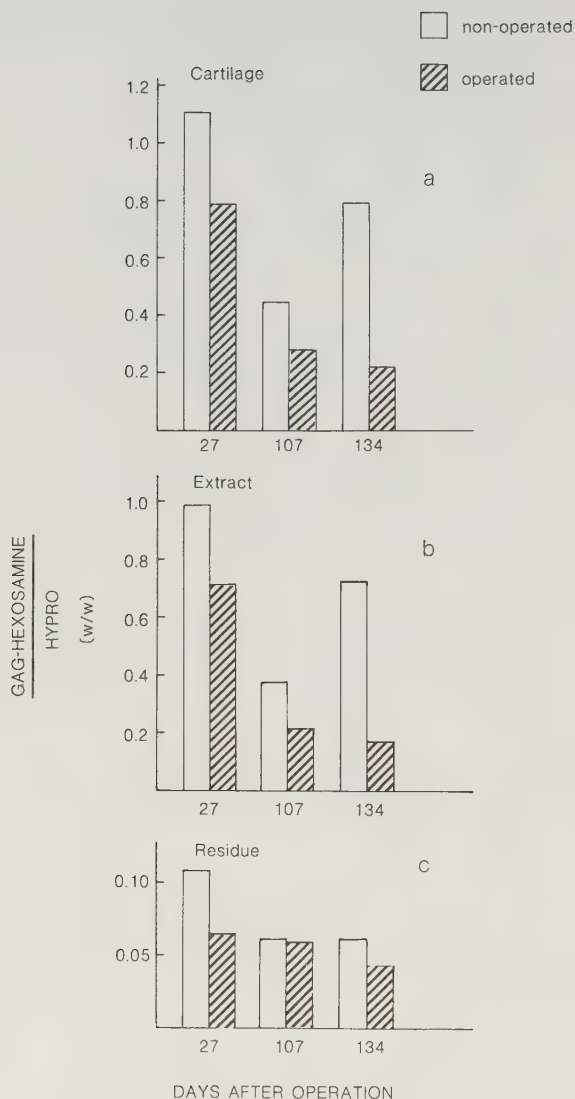


Figure 7. Proteoglycan contents at various times after knee surgery. Proteoglycans in the cartilage (a), extract (b) and residue (c) after extraction with 4 M guanidinium chloride. Proteoglycans quantified as glycosaminoglycan-hexosamine over hydroxyproline (w/w).

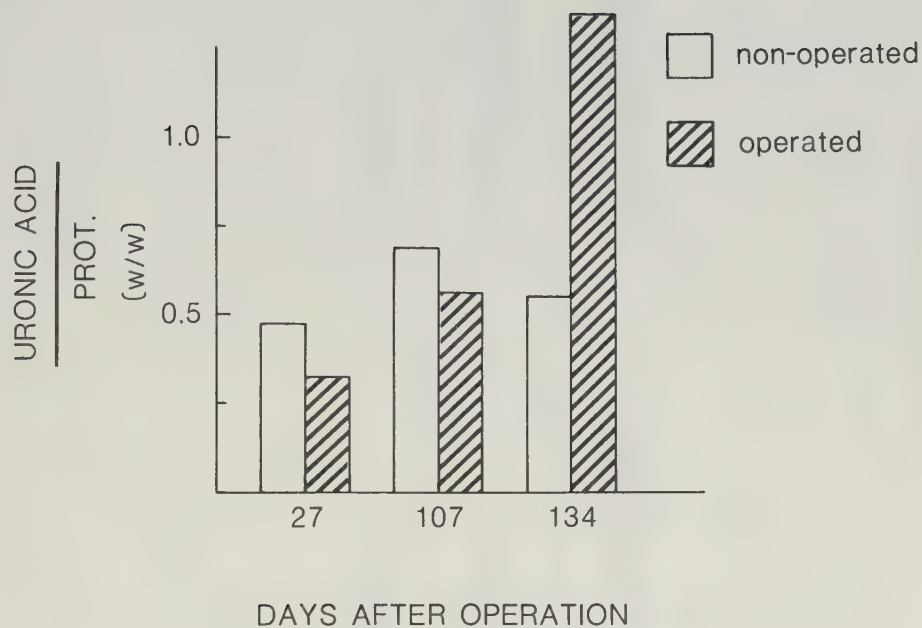


Figure 8. Uronic acid to protein ratio (w/w) of isolated proteoglycans A1-fractions at various times after knee surgery.

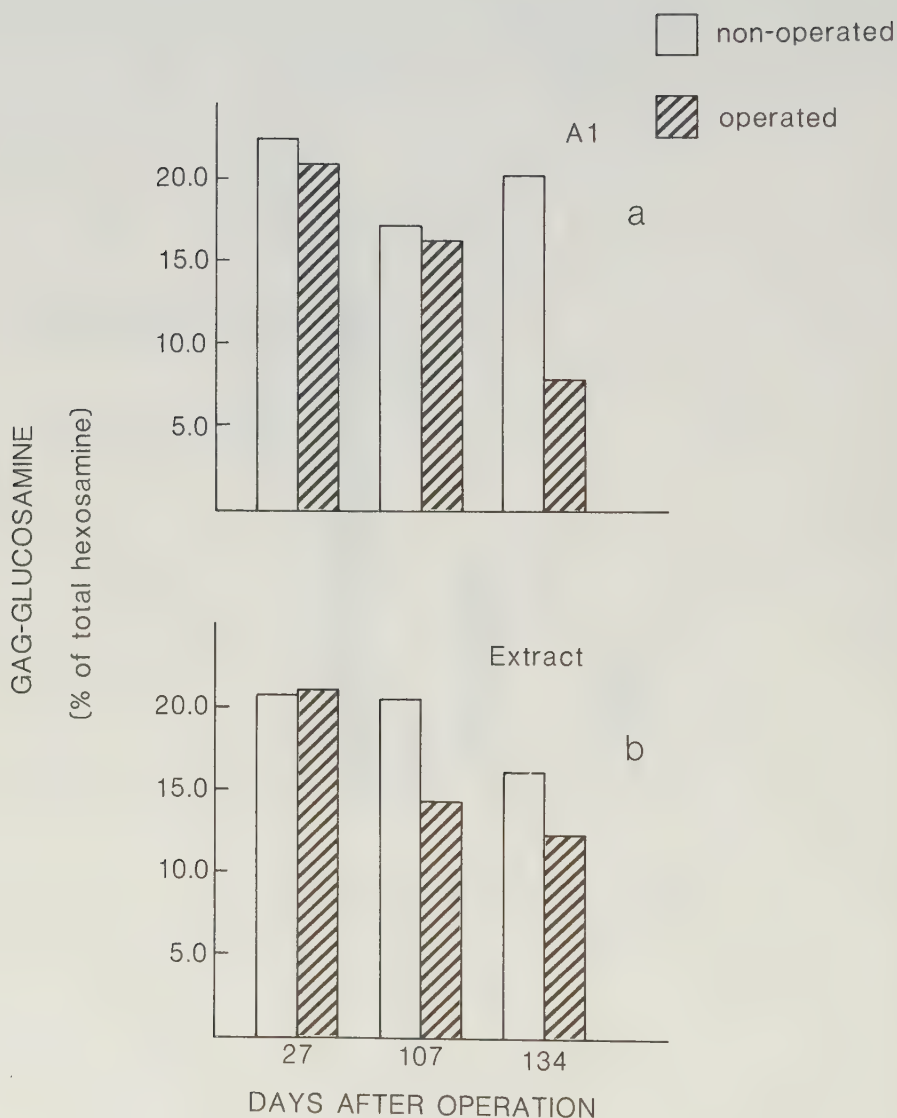


Figure 9. Ratios of glycosaminoglycan-glucosamine (keratan sulfate) to total glycosaminoglycans in isolated proteoglycans (A1) (a) and in guanidinium chloride extracts (b) of knee articular cartilage after transection of the anterior cruciate ligament.

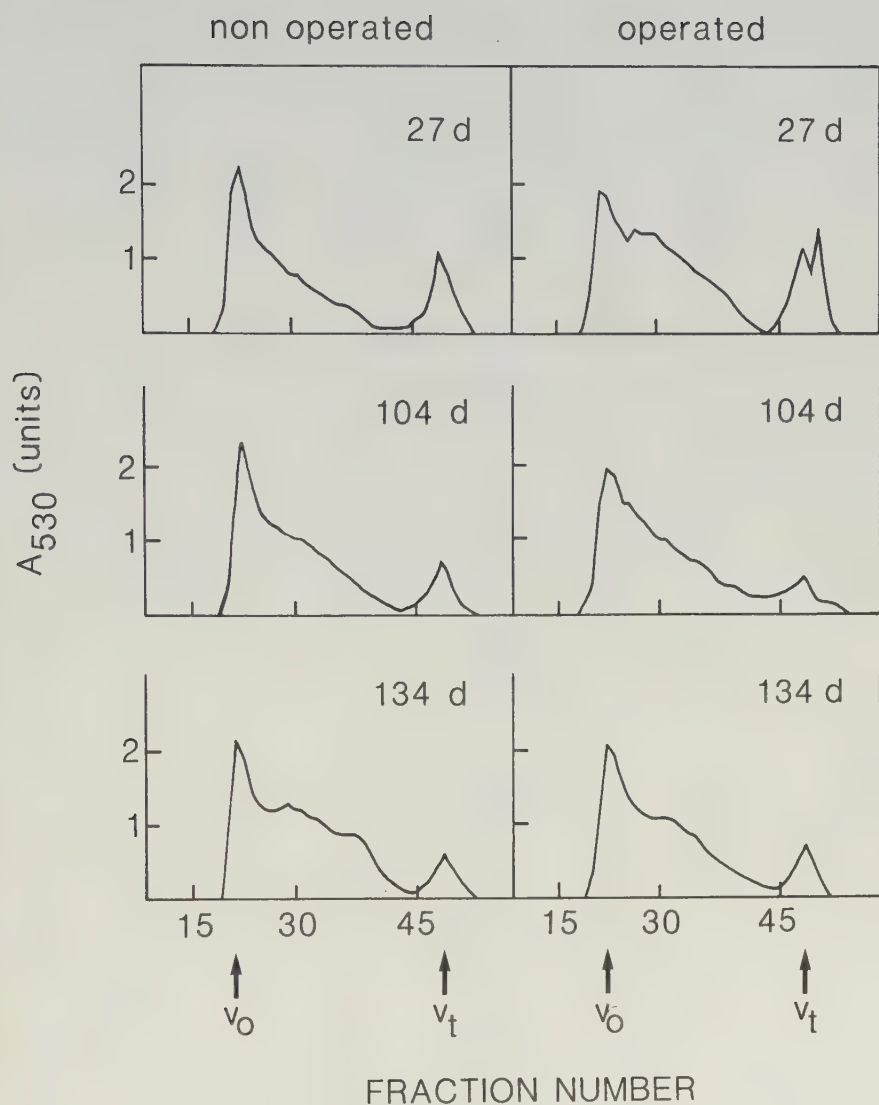


Figure 10. Chromatography on Sepharose 2B of the A1-fractions from the dogs operated on with transection of the anterior cruciate ligament. Operated and non-operated sides shown to the right and left, respectively. The time intervals after surgery are from top to bottom 27, 107 and 134 days. The absorbance (A_{530}) of the carbazole reaction is given as arbitrary units.

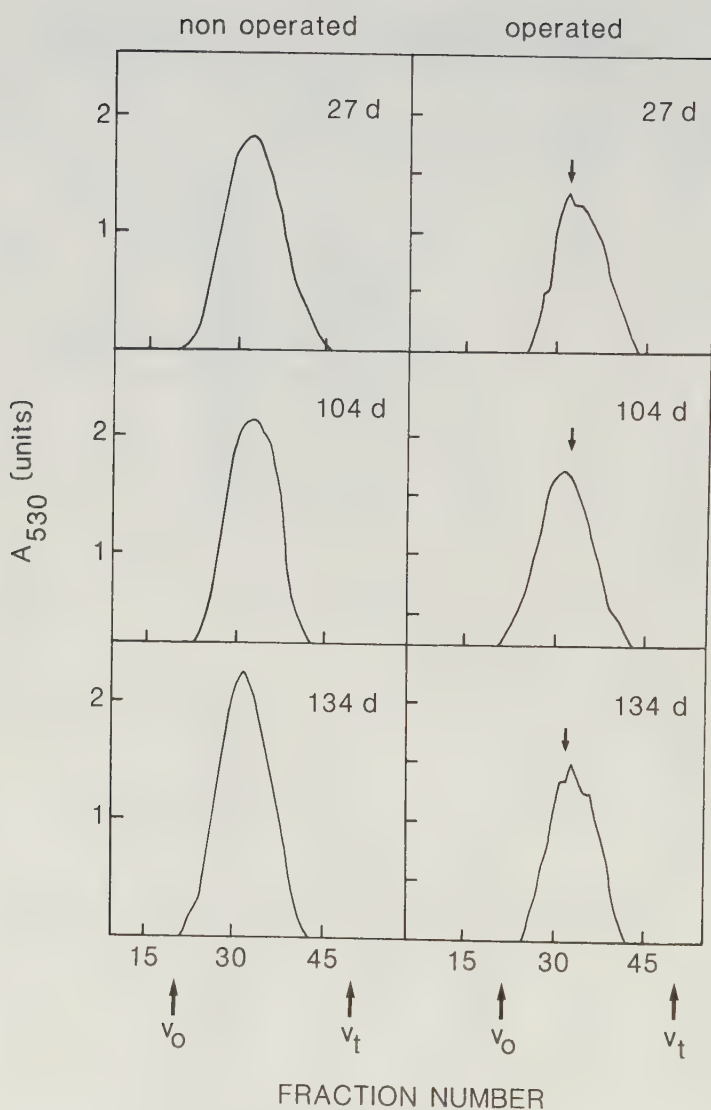


Figure 11. Chromatography on Sepharose 2B of reduced and alkylated A1-fraction from the dogs operated on with transection of the anterior cruciate ligament. Operated and non-operated sides shown to the right and left, respectively. The time intervals after surgery are from top to bottom 27, 107 and 134 days. The arrows in the chromatograms from the side operated on show the position of the peak of the control side. The absorbance (A_{530}) of the carbazole reaction is given as arbitrary units.

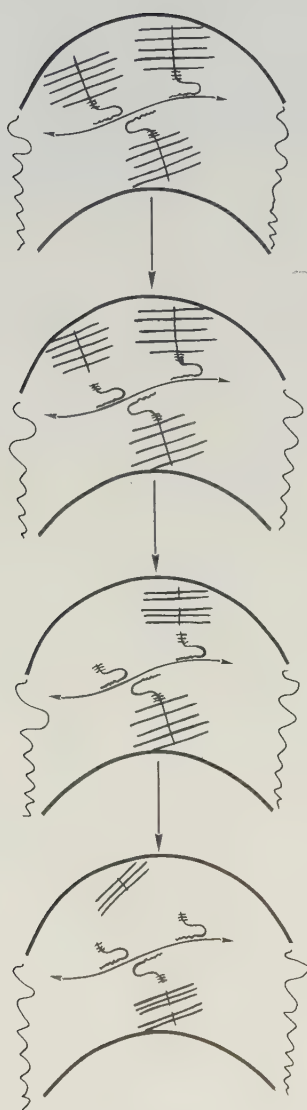


Figure 12. Tentative changes in the structure of cartilage proteoglycans in developing hip osteoarthritis.

THE NORMAL VARIATION IN STRUCTURE AND COMPOSITION OF CANINE HIP
ARTICULAR CARTILAGE PROTEOGLYCANS

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ABSTRACT

The composition of the weight bearing portion of the femoral head articular cartilage from both sides of greyhounds 11-32 months of age was studied with regard to variability of proteoglycans. Extractability of the proteoglycans was very similar for all samples. Other parameters, i.e. contents of proteoglycans in the cartilage, hyaluronic acid content of the proteoglycan fraction as well as its keratan sulfate and chondroitin sulfate contents, size of proteoglycans and their aggregatability with hyaluronate, were similar in samples from the right and left side of each dog, while the variability from one dog to another was large and not related to age. The data show that in studies of joint disease the contralateral joint can be used as a control to the treated joint, while the interindividual variability precludes comparison between individuals.

INTRODUCTION

Articular cartilage contains about 80% water and 20% organic material. Some 70% of the organic material is collagen and 25% is proteoglycan (Lipshitz et al., 1975). The remaining 5% consists of cartilage matrix proteins and cellular components. The collagen fibers, provide tensile strength (Kempson et al., 1973) and the proteoglycans convey compressive stiffness or more generally, elasticity, properties that are essential for the function of the cartilage (Kempson et al., 1970; Harris et al., 1972). The proteoglycans are large macromolecules with molecular weights of $1.1-1.6 \times 10^6$ (Rosenberg, 1975; Swann et al., 1972). They contain a central protein core to which polysaccharide chains of chondroitin sulfate and keratan sulfate are covalently attached. The chondroitin sulfate chains are attached to a region at one end of the core protein, the chondroitin sulfate rich region (Heinegård & Axelsson, 1977). The keratan sulfate chains are also concentrated to a portion of the core protein, the keratan sulfate rich region, which is located between the chondroitin sulfate rich region and a globular region forming the other end of the proteoglycan core protein. This, hyaluronic acid binding region contains no glycosaminoglycan chains. It has a specific affinity for hyaluronic acid (Heinegård & Hascall, 1974) and mediates aggregation of proteoglycans to hyaluronic acid. Thereby proteoglycan aggregates with molecular weights of the order of 100×10^6 are formed (Hardingham & Muir, 1973). Recently Heinegård et al. (1981a) have identified discrete subpopulations of aggregating proteoglycans, one chondroitin sulfate rich subpopulation of high molecular weight and one keratan sulfate rich subpopulation of somewhat lower molecular weight. Furthermore, cartilage contains a high molecular weight nonaggregating proteoglycan, as well as a nonaggregating low molecular weight, protein rich proteoglycan (Heinegård et al., 1981b).

In aging the proportion between the two high molecular weight proteoglycan subpopulations changes (Stanescu, 1978; Inerot & Heinegård, 1983). At older age the keratan sulfate rich subpopulation is relatively more abundant compared with the chondroitin sulfate rich subpopulation. Furthermore, the average molecular size of the proteoglycan monomers is lower at old age, partly explained by the larger proportion of the smaller proteoglycan (Inerot et al., 1978; Bayliss & Ali, 1978; Sweet et al., 1979; Roughley & White, 1980; Inerot and Heinegård, 1983). As a result of changing proportions of subpopulations, contents of protein and keratan sulfate is higher in proteoglycans from older cartilage while the content of chondroitin sulfate is lower (Inerot & Heinegård, 1983).

In degenerative joint disease, such as osteoarthritis, the content of proteoglycans in the cartilage is reduced. For the further study of the early development of osteoarthritis, experimental models have been used (for references see Olsson et al., 1983). A model of hip osteoarthritis which has the advantage of not primarily interfering with joint structures has been developed. In this model a secondary osteoarthritis is induced in greyhounds through extra-articular pelvic osteotomies, which produces a hip dysplasia like condition. Degeneration of the articular cartilage follows within months after surgery (Olsson et al., 1983).

In most experimental models of arthritis, articular cartilage from one affected side, usually operated on, is compared with the articular cartilage of the contralateral joint. An essential background for the interpretation of the results obtained, is knowledge of the interindividual as well as the intraindividual variability with regard to structural macromolecules, i.e. differences between individuals with regard to a particular joint and difference in each individual between a joint on one side and the contralateral joint, respectively.

The present study was initiated to provide such background information, with main focus on proteoglycans. The hip joint of greyhounds was chosen, because of our work with the model of hip osteoarthritis discussed above.

MATERIALS AND METHODS

Femoral head articular cartilage samples from the area of maximum weightbearing, just dorsal to the ligamentum teres, was obtained from ten greyhounds of racing lineage 11-32 months of age. The dogs were sacrificed through intravenous injection of large doses of sodium barbital. The hip joints of both sides were immediately opened, the articular surface was blotted with a paper napkin and the cartilage samples were removed with a scalpel as described elsewhere (Inerot et al., 1983). All hip joints had normal macroscopic appearance and there were no signs of cartilage degeneration. The cartilage samples were immediately frozen in liquid nitrogen and stored at -60°C until further processed.

Prior to extraction the frozen cartilage samples were sliced in a cryotome in 20 µm thin sections, which were pooled and stored at -60°C until extraction. Care was taken to avoid thawing of the tissue during handling. The sliced cartilage samples, each containing approx. 50 mg of cartilage, were extracted for 24 hrs at 40°C with 2 ml of 4 M guanidinium chloride, 0.05 M sodium acetate, pH 5.8 also containing the protease inhibitors used by Oegema et al. (1975). Extracts and residues were separated as described previously (Franzén et al., 1981).

The glycosaminoglycans were isolated by ion exchange chromatography on DEAE cellulose of papain digests of extracts and residues as described by Axelsson & Heinegård (1975).

CsCl density gradient centrifugation in 4 M guanidinium chloride using a starting density of 1.35 g/ml (Heinegård *et al.*, 1981b) was used to purify proteoglycans, which were recovered in the bottom third of the gradient (dD1-fraction). Proteoglycan samples were chromatographed on Sepharose 2B after reduction and alkylation (Inerot *et al.*, 1978) to estimate the hydrodynamic volume of the proteoglycan monomers. The ability of the proteoglycans to interact with hyaluronic acid was assessed by chromatography on Sepharose 2B after the sample was incubated overnight at 40°C with 2% (w/w) of high molecular weight hyaluronic acid (Healon^R, Pharmacia AB, Uppsala, Sweden). The aggregates chromatograph in the void volume while the nonaggregating monomers elute in the included volume.

The molecular size of the chondroitin sulfate chains was determined by chromatography on Sephadex G200 of papain digests of proteoglycans (Inerot *et al.*, 1978).

Agarose-polyacrylamide gel electrophoresis was performed essentially according to McDevitt & Muir (1971) with minor modifications to prevent aggregation by including SDS in the buffers (Heinegård, unpublished). The gels were stained with toluidine blue.

The glycosaminoglycans in the proteoglycan dD1-fraction were isolated by chromatography on DEAE-cellulose after alkaline borohydride treatment as described previously (Inerot & Heinegård, 1983). The content of uronic acid and protein were determined by automated versions of the carbazole and Folin procedures (Heinegård, 1973). Content of hexosamines were determined on a Durrum amino acid analyzer after hydrolysis of samples in 4 M HCl (Aristar grade) under argon at 100°C for 10 hrs. Hydroxyproline contents in extracts and residues were determined by the method of Stegeman and Stalder (1967) after hydrolysis of samples in 6 M HCl in sealed tubes for 24 hrs at 100°C. Content of hyaluronic acid in dD1-fractions was determined after treatment of samples with 0.1M sodium hydroxide for 40 hrs at 20°C. A radioligand method using ¹²⁵I-labeled hyaluronic acid binding region was used (Wieslander & Heinegård, unpublished).

RESULTS AND DISCUSSION

Composition of cartilage

Contents of proteoglycans in the cartilage was related to contents of collagen (hydroxyproline), the latter assumed to show little variability. Total amounts of glycosaminoglycan-hexosamines in extract plus residue, showed only minor differences between the right and the left hip joint of most individuals. The largest difference was about 15% of the higher value, Table I. The interindividual variation, on the other hand, was considerable, *i.e.* the lowest value observed was only about half that of the highest, Table I.

Cartilage samples were extracted with 4M guanidinium chloride. The extraction yield was high for all samples, *i.e.* 38-97% of the proteoglycans were extracted, Table I. The variation within the individual was small, and the yield was essentially the same in all individuals.

Further analysis of extracts and residues, respectively, showed very small differences in the ratio of glucosamine to galactosamine of glycosaminoglycans comparing left to right hip joint, while the interindividual variations were marked, Table I.

Composition of isolated proteoglycans

Proteoglycans were isolated from the extract by using centrifugation in 4M guanidinium chloride-CsCl at a low starting density. The bottom fraction contains the bulk of the proteoglycans, including those with high protein contents (Heinegård *et al.*, 1981b), as well as some hyaluronic acid.

To characterize these proteoglycans, the following molecular constituents were measured (the structure represented indicated within brackets): protein (protein core), uronic acid (chondroitin sulphate plus hyaluronate), galactosamine in glycosaminoglycans (chondroitin sulphate) and glucosamine in glycosaminoglycans (keratan sulphate plus hyaluronate). Furthermore hyaluronate was quantified separately.

Contents of hyaluronic acid showed minor differences between joints of the same individual, while variation from one individual to another was more pronounced, Table II. The contribution of hyaluronic acid to the glucosamine values as well as to the uronic acid values of the preparations is very low and furthermore relatively constant within the individual. Therefore, the comparatively small differences in relative glycosaminoglycan-glucosamine contents between right and left hip, can be taken to indicate that contents of keratan sulphate showed little variability within the individual, Table II. The variations from one individual to another, however, were large (range 0.114 to

0.208). The somewhat older individuals showed the higher values, in accordance with published data (Inerot et al., 1978; Bayliss & Ali, 1978; Sweet et al., 1979; Roughley & White, 1980 and Inerot & Heinegård, 1983).

The ratio of chondroitin sulphate (uronic acid) to protein was also similar for samples from the left and the right hip joint, respectively, Table II. This ratio was high (0.8) in a few individual, while the major portion of the samples had a similar ratio of about 0.5. As discussed above contribution by hyaluronic to the uronic acid contents can be neglected.

Properties of proteoglycans

Monomers: The size distribution of the proteoglycan monomers was determined by chromatography on Sepharose 2B, after previous reduction of samples, Fig 1. The elution profiles of the proteoglycans from the right and left hip joint of the same individual was very similar or identical. Some variability was noted from one individual to another, Fig. 1.

Subpopulations of proteoglycan monomers were studied by agarose-polyacrylamide gel electrophoresis, Fig. 2. Two major components, corresponding to the large proteoglycans, were present in similar proportions and showed the same electrophoretic mobility in samples from each individual. Between individuals, however, the pattern could be quite different. An additional, minor, component of high electrophoretic mobility was also observed in all samples, probably corresponding to the low molecular weight cartilage proteoglycan (Stanescu & Sweet 1981; Heinegård et al., 1981b).

Aggregatability: The formation of aggregates of the proteoglycans (dD1) and the added hyaluronic acid (2%, w/w) was studied, by gel chromatography of the mixture, Fig. 3. The proportion of aggregates, eluting in the void volume, was generally more similar for samples from the same individual than for samples from different individuals.

Size of chondroitin sulphate chains: Proteoglycan preparations (d•D1) were digested with papain to free the chondroitin sulphate chains. Subsequent chromatography on Sephadex G-200 showed very similar elution profiles for all samples, i.e. essentially neither intra- nor interindividual variation, Fig. 4.

GENERAL DISCUSSION

In the present investigation, hip articular cartilage from only greyhounds of a narrow age span have been studied. Therefore, one should not expect variation due to age. Expectedly, then, there is no trend, unlike a particular parameter increases or decreases with age. The differences between individuals with regard to most parameters studied are marked. Indeed the variation between individuals is so large that it would probably mask many alterations caused by a process in the joint of an affected animal if compared with a control animal. Fortunately, however, the samples taken from the right and the left hip joint, respectively, were quite similar with regard to all parameters studied. Therefore, in studies of effects of therapy or in studies of joint disease, the contralateral joint can serve as an adequate control.

The small differences between the two sides are not consistently such that one particular side always has higher or lower values. Rather the differences observed are usually within the error of the methods used.

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Table 1. Glycosaminoglycan (GAG) content and composition of articular cartilage, extract and residue.

Age (months)	Side (left or right)	<u>GAG Hexosamine</u> hydroxyproline in cartilage (w/w)	Extraction yield (%)	<u>GAG-glucosamine</u> <u>GAG-hexosamine</u> (w/w)		Cartilage
				Extract	Residue	
11	right	0.70	94	0.124	0.115	0.123
	left	0.68	90	0.136	0.118	0.134
12	right	0.51	93	0.132	0.105	0.130
	left	0.57	92	0.141	0.115	0.138
15	right	0.52	96	0.143	0.135	0.143
	left	0.55	92	0.136	0.124	0.135
15	right	0.61	90	0.153	0.123	0.150
	left	0.61	95	0.157	0.136	0.156
21	right	0.86	89	0.196	0.150	0.191
	left	0.79	93	0.210	0.154	0.206
23	right	0.49	93	0.202	0.186	0.200
	left	0.58	97	0.189	0.184	0.189
26	right	0.61	95	0.194	0.174	0.193
	left	0.56	90	0.181	0.169	0.180
28	right	0.64	94	0.185	0.157	0.184
	left	0.56	95	0.199	0.161	0.197
32	right	0.58	88	0.177	0.152	0.174
	left	0.61	90	0.165	0.154	0.164
32	right	0.42	91	0.180	0.175	0.179
	left	0.41	89	0.173	0.170	0.173

Table 2. Relative contents of uronic acid, protein, glycosaminoglycan (GAG) hexosamine and glucosamine in proteoglycan (dD1) fractions. Also shown are contents of hyaluronic acid.

Age (months)	Side (right or left)	<u>GAG glucosamine</u> GAG hexosamine (w/w)	<u>Uronic acid</u> protein (w/w)	<u>Hyaluronic acid</u> uronic acid (w/w)
11	right	0.114	0.57	0.020
	left	0.138	0.61	0.018
12	right	0.130	0.55	0.019
	left	0.143	0.56	0.020
15	right	0.139	0.80	0.015
	left	0.121	0.75	0.020
15	right	0.146	0.65	0.019
	left	0.153	0.60	0.025
21	right	0.198	0.50	0.024
	left	0.208	0.45	0.024
23	right	0.172	0.49	0.014
	left	0.162	0.53	0.017
26	right	0.188	0.45	0.031
	left	0.180	0.42	0.025
28	right	0.168	0.50	0.033
	left	0.177	0.52	0.033
32	right	0.172	0.50	0.024
	left	0.162	0.50	0.020
32	right	0.175	0.53	0.024
	left	0.153	0.49	0.018

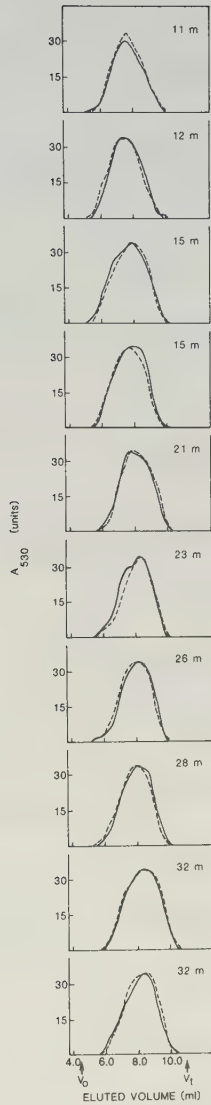


Fig. 1. Sepharose 2B chromatograms of reduced and alkylated proteoglycan monomers. The uronic acid profiles (carbazole procedure) from the right (----) and left (—) hip joint at the ages of (from top to bottom) 11, 12, 15, 15, 21, 23, 26, 28, 32 and 32 months are shown. The chromatography was performed on a 140 x 0.3 cm column eluted with 0.5 M sodium acetate, pH 7.

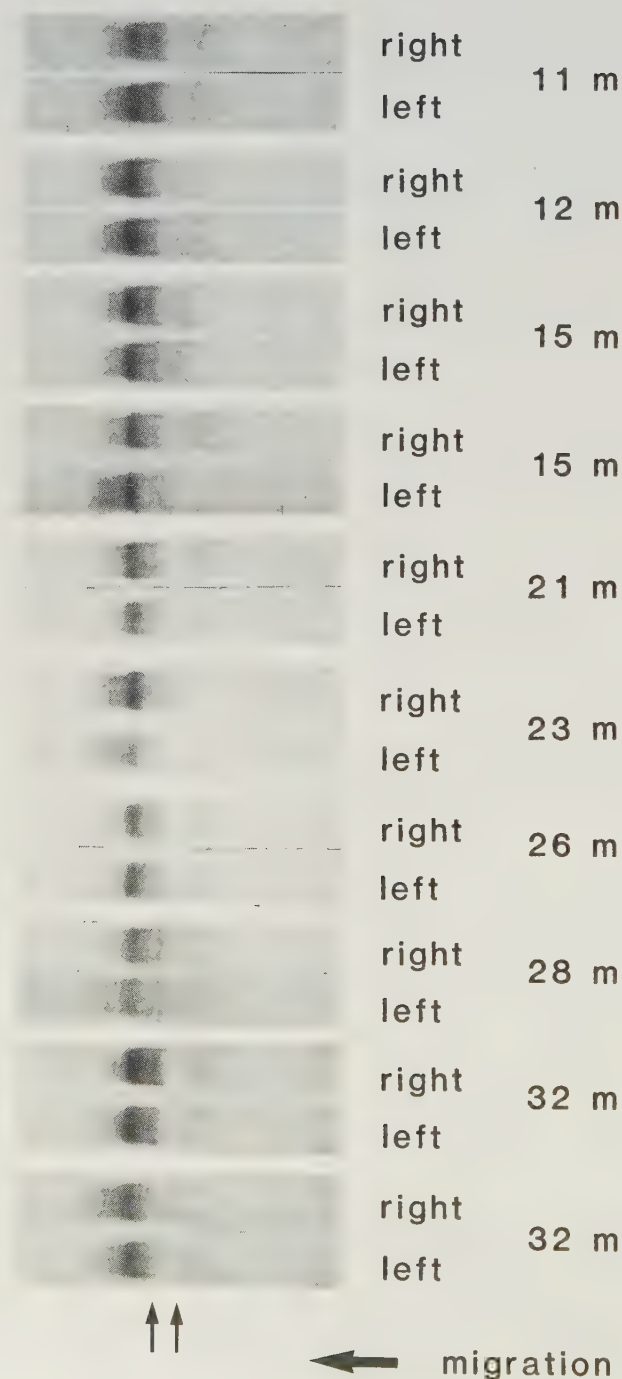


Fig. 2. Agarose-polyacrylamide gel electrophoresis of dD1-fractions. The migration positions of keratan sulfate rich proteoglycans and chondroitin sulfate rich proteoglycans of a reference preparation from bovine tracheal cartilage are indicated with arrows.

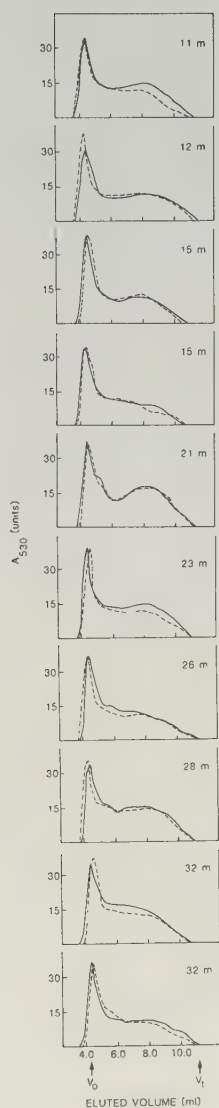


Fig. 3. Sepharose 2B chromatograms of proteoglycan fractions after incubation with 2% hyaluronic acid. The uronic acid profiles (carbazole procedure) from the right (-----) and left (——) hip joint at the ages of (from top to bottom) 11, 12, 15, 15, 21, 23, 26, 28, 32 and 32 months are shown. The chromatography was performed on a 140 x 0.3 cm column. The eluting buffer was 0.5 M sodium acetate, pH 7.0, also containing 0.5 μ g/ml of human umbilical cord hyaluronic acid (Sigma Chemical Co, St Louis, MO).

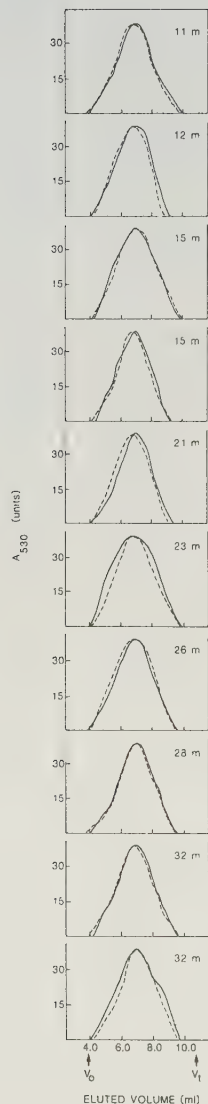


Fig. 4. Sephadex G200 chromatograms of papain-digested dD1-fractions. The uronic acid profiles (carbazole procedure) from the right (---) and left (—) hip joints at the ages of (from top to bottom) 11, 12, 15, 15, 21, 23, 26, 28, 32 and 32 months are shown. The chromatography was performed on a 140 x 0.3 cm column eluted with 0.5 M sodium acetate, pH 7.0.

QUANTITATION OF CARTILAGE PROTEOGLYCAN STRUCTURES LIBERATED TO
THE SYNOVIAL FLUID DURING DEVELOPING DEGENERATIVE JOINT DISEASE

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Abstract

An enzyme linked immunosorbent assay for quantitation of proteoglycans or proteoglycan fragments in biological fluids is presented. The assay has been used to determine concentrations of articular cartilage proteoglycans and/or fragments thereof in synovial fluid in early stages of osteoarthritis.

Osteoarthritis was induced in one knee joint of 9 German wire haired pointers by transection of the anterior cruciate ligament. All dogs developed osteoarthritis by macroscopic as well as microscopic criteria. Attempts were made to aspirate synovial fluid, prior to surgery and at various times in the postoperative period, from the joint operated on as well as from the contralateral control joint. Concentrations of proteoglycans (fragments) were significantly higher in the synovial fluid samples from the joint operated on compared with the control joint ($p < 0.001$ and $p < 0.1$ for two types of aggregating proteoglycans, respectively). The concentration of proteoglycans (fragments) in synovial fluid samples from joints operated on was higher after the operation compared with the preoperative concentration. No correction has been made for the increased dilution due to hydrops of the joints operated on.

Introduction

Articular cartilage has a high content of proteoglycans, which contribute fixed negative charges, that are important for the normal function of the tissue (6).

The proteoglycans consist of a central protein core containing covalently bound, strongly polyanionic glycosaminoglycan side chains of chondroitin sulphate and keratan sulphate. Three regions of the protein core can be identified (1), one containing no glycosamino-glycan chains, the hyaluronic acid binding region, one containing keratan sulphate chains, the keratan sulphate rich region, and the major portion of the molecule containing primarily chondroitin sulphate chains and a few keratan sulphate chains, the chondroitin sulphate rich region. Recently it has been shown that there are two antigenically different populations of large proteoglycans in cartilage, i.e. the chondroitin sulphate rich proteoglycan and the keratan sulphate rich proteoglycans (2).

Destruction of the articular cartilage is a typical finding in degenerative joint disease, i.e. osteoarthritis. The earliest event that can be detected histologically is fibrillation and loss of metachromasia when the tissue is stained with toluidine blue. This loss of metachromasia is explained by a decreasing content of proteoglycans in the cartilage matrix. In late stages the surface of the articular cartilage is eroded due to loss of tissue (for ref. see 3). Patients do not seek care until the degenerative process is well advanced, since clinical symptoms occur late. Therefore in order to allow studies of early events a number of animal models for osteoarthritis have been developed. Frequently used models involve induction of an unstable knee joint, most commonly by transection of the anterior cruciate ligament (10). The unstable joint develops osteoarthritis. Another model involves changing the mechanical factors of the hip joint of dogs by osteotomy of the pelvic bones in an attempt to mimic hip dysplasia with ensuing osteoarthritis (9). Studies of the articular cartilage at different times after the operation have revealed that decreased contents of proteoglycans is an early event. The likely mechanism is proteolytic fragmentation of the proteoglycans allowing liberated fragments to diffuse out of the cartilage. In support, fragments of proteoglycans, probably formed by proteolysis of the intact molecules, have been identified in the osteoarthrotic cartilage (4).

The proteoglycan fragments are only free to diffuse to the synovial fluid, as the bone-cartilage border forms an impermeable barrier (8). Some work (11) has been aimed at identifying, in synovial fluid, products of increased catabolism of articular cartilage. A major problem has been to identify the source of the macromolecular components in the synovial fluid. Immunological

techniques, however, allow specific, sensitive quantitation of cartilage proteoglycans and fragments thereof (13). The present investigation was therefore undertaken to demonstrate if contents of cartilage proteoglycan antigens, increased in synovial fluid as an indication of cartilage destruction during developing osteoarthritis. It was considered important to follow also the very early events. Therefore we chose to use one of the animal models of knee osteoarthritis (10), that allows synovial fluid samples to be aspirated at various times.

Materials and Methods

Preparation of antigens. Proteoglycans were extracted from bovine nasal and canine hip articular cartilage, respectively, and purified by CsCl-density gradient centrifugation under the dissociative conditions of 4 M guanidinium chloride (2). The subpopulations of chondroitin sulphate rich (D1-S1) and keratan sulphate rich (D1-S2) proteoglycans of bovine nasal cartilage, were prepared as described elsewhere (2). Proteoglycan preparations to be used for immunization were purified by chromatography on Sephadex G-200 eluted with 4 M guanidinium chloride (12).

Before their use as antigen in the assay, proteoglycans were digested with chondroitinase ABC to remove chondroitin sulphate side chains. Samples were dissolved in 0.05 M Tris-HCl, pH 8.0, at 1 mg/ml and digested for 6 h at 37°C. Dilutions of the digests were used without further treatment.

Preparation of synovial fluid samples. Nine German wire haired pointers were kept in the animal room for 40-75 days prior to surgery. At the day of surgery, synovial fluid samples were taken from both knee joints and the anterior cruciate ligament of the left joint was transected according to McDevitt et al., (7). The dogs began to use the operated leg after 4-10 days. After 3, 6, 10, 15, 19, 26 and 29 weeks samples of synovial fluid were aspirated from the left, operated, as well as from the right, control joint. All dogs were killed 7 months after surgery and the joint operated on was opened to disclose degenerative changes.

Occasionally the attempts to aspirate synovial fluid were unsuccessful. A further limitation was that the original purpose of the investigation necessitated the use of much of the synovial fluid samples for other types of analyses. Remaining samples were made available for the present study. Only synovial fluid samples taken from both the left and the right knee joint of a particular dog on the same occasion were analyzed. In addition, all samples from the left knee joints were analyzed, provided that a preoperative sample was available. All samples were stored at -60°C. Prior to analyses all samples were digested with chondroitinase

ABC (an early batch not containing proteolytic activity) in order to depolymerize the hyaluronate and remove the chondroitin sulphate side chains of the proteoglycans. A predigestion to reduce the viscosity of the synovial fluid was performed by adding 2 μ l (< 1% of the sample volume) of a solution containing 0.01 (nominal) units of chondroitinase in 1.25 M Tris-HCl, pH 8.0. After 4 h at 37°C, aliquots (50 μ l) were removed and mixed with 400 μ l of 0.1 M Tris-HCl, pH 8.0, containing 0.01 unit of chondroitinase ABC. After incubation at 37°C for a further 4 h samples were frozen until analysis.

Antisera. Antibodies were raised in rabbits immunized with intact canine articular cartilage proteoglycan monomer. Initial injection of 1.5 mg of proteoglycan monomer in Freund's complete adjuvant was followed by two monthly injections of 1.0 mg of proteoglycan in Freund's incomplete adjuvant. At this time the antibody titer was sufficient for use in immunoassay. The serum was not purified before use in the assay.

Enzyme-linked immunosorbent assay (ELISA). Dynatech (M 29) polyvinyl microtiter plates were used. The wells were coated (24 h, room temperature) with 200 μ l of bovine chondroitin sulphate rich proteoglycan (2 μ g/ml; D1-S1) or 200 μ l of bovine keratan sulphate rich proteoglycan (0.5 μ g/ml; D1-S2) (2). Stock solutions (0.5 mg/ml in 0.05 M Tris-HCl, pH 8.0) of these proteoglycan preparations were digested with chondroitinase ABC (0.01 units/mg) for 4 h at 37°C prior to further dilution with the Tris-buffer and subsequent use for coating. The plates were extensively rinsed with 0.15 M sodium chloride, 0.05% Tween 20 to remove unbound proteoglycan. Synovial fluid samples (50 μ l, chondroitinase digested) were diluted with 750 μ l of 0.10 M sodium chloride, 0.05 M sodium phosphate, 0.05% (w/v) Tween 20, pH 7.5. Samples (110 μ l) of the dilutions were mixed with equal samples (110 μ l) of a dilution 1/150, in the same buffer, of the rabbit anti-canine articular cartilage proteoglycan serum. After preincubation for 24 h at room temperature 200 μ l of the mixture was added to the coated wells of the microtiter plate. After incubation for 60 min at room temperature, the plates were rinsed as above and 200 μ l of a dilution (1/150) of swine-anti rabbit IgG conjugated with alkaline phosphatase (Orion Diagnostica, Helsinki, Finland) in 0.10 M sodium chloride, 0.05 M sodium phosphate, 0.05% Tween 20, 2 mg/ml of bovine serum albumin, pH 7.5, was added. After 60 min at room temperature the plates were again rinsed and 200 μ l of enzyme substrate, 1 mg/ml of p-nitrophenylphosphate in 1 M diethanolamine, pH 9.8, containing 0.5 mM $MgCl_2$, was added. Absorbance at 405 nm was measured in a Titertec Multiscan filterphotometer (Flow Laboratories, U.S.A.), immediately and after 60 min of incubation at room temperature. The increase in absorbance was used for calculations. A standard curve of canine articular cartilage proteoglycan monomer (digest-

ed with chondroitinase ABC) was included in each microtiter plate. All samples were analyzed in triplicate and the mean value was used for calculations using a spline function. t-Statistics was calculated using standard programs for a Hewlett-Packard (Corvallis, Oregon, U.S.A.) 41CV calculator.

Results and discussion

Enzyme Linked Immunosorbent Assay (ELISA). One major problem in ELISA is variable results due to variations in coating as a result of the surface properties of the plastic (5). To achieve optimal results the best plastic for use with each particular antigen has to be found. One important consideration is to achieve high capacity in the assay. Therefore in the present study we tested only tubes and microtiter plates that could be used directly in automated photometers. Two different types of procedures were tested: 1) the reactions were performed in disposable polystyrene cuvettes for the LKB-2074 photometer and 2) the reactions were performed in microtiter plates of polystyrene (Nunc Immunoplate and Dynatech) or polyvinyl (Dynatech M 29). Using various proteoglycan antigens, the most reproducible results were always obtained with the polyvinyl microtiter plates. Using these plates the c.v. of the ELISA within one plate varied from 3 to 5%. Using polystyrene plates, regardless of manufacturer, the c.v. of the ELISA varied from about 10% to about 25% depending on the antigen used. The polystyrene cuvettes gave somewhat better results with a c.v. of the assay of 5%. It was concluded that acceptable results were only obtained with polyvinyl microtiter plates or polystyrene tubes. The microtiter plates allowing higher capacity were used for all subsequent experiments. It is possible that coating of much higher concentrations of antigen may improve reproducibility, but at the cost of sensitivity.

ELISA was done using homologous assays i.e. dog proteoglycans for coat, competitor and antigen for immunization (rabbit anti-dog proteoglycan antibodies), as well as using heterologous assays i.e. bovine nasal cartilage proteoglycans for coat and dog articular cartilage proteoglycans as competitors, in combination with rabbit anti dog proteoglycan antibodies. The sensitivity of the heterologous assay was somewhat higher. As a large spectrum of bovine nasal cartilage proteoglycan antigens was available, heterologous ELISA was preferred. The sensitivity of the ELISA was considerably increased when the competing antigens were preincubated overnight with the antibody and the mixture subsequently only incubated for a short time (60 min) in the coated wells. The sensitivity of the ELISA for D1-S2 was somewhat greater than that for D1-S1, Fig. 1.

Development of osteoarthritis. All dogs developed osteoarthritis. Three months after surgery, x-ray showed osteophytes (data not shown). At the time of death all dogs had macroscopic degenerative changes of the articular cartilage of the joint operated on, i.e. yellow discoloration, pitting of the surface or erosion. The medial meniscus was damaged and osteophytes and synovitis, i.e. thickened synovial membrane and proliferative changes were seen on microscopy.

Synovial fluid. Samples of synovial fluid were more frequently recovered from the left joint, which had been operated on, mainly because of hydrops. The present study includes samples from the left and right knee joint aspirated on the same occasion as well as samples from the left (operated) joint of dogs where also a preoperative sample had been obtained.

A total of 27 samples from the left and the right joints, respectively, were recovered from 8 dogs. The number of duplicate (left plus right) samples aspirated at 3, 6, 10, 15, 19, 26 and 29 weeks were 3, 5, 5, 4, 4, 3 and 3, respectively. 29 sequential samples including the preoperative sample were recovered from 4 dogs, Fig. 2.

Provided that proteoglycan fragments are liberated to the synovial fluid at a constant rate, the concentration of fragments will depend on the volume of the fluid. In case of hydrops, therefore, the concentration of proteoglycan fragments in the fluid will be low. In the present investigation no attempt has been made to correct for this dilution due to hydrops, which being pronounced at the side operated on might obscure increased liberation of proteoglycan fragments.

Concentration of proteoglycan antigens. In an attempt to differentiate between different types of antigens released from the cartilage to the synovial fluid, ELISA was done using coat of either chondroitin sulphate rich proteoglycans (D1-S1) or keratan sulphate rich proteoglycans (D1-S2). Indeed the results were somewhat different. The contents of proteoglycan antigens are expressed as proteoglycan equivalents, i.e. the amount of the dog articular cartilage proteoglycans giving the same inhibition in the ELISA, Table I and II. The concentration of proteoglycan antigenic components in the synovial fluid of the left joint was significantly higher than that of the right joint at the same occasion ($p < 0.001$ for D1-S1 and $p < 0.1$ for D1-S2) showing increased liberation of proteoglycan antigens during developing osteoarthritis. Interestingly, the results obtained with the two antigens differed. It appears that more of the chondroitin sulphate rich proteoglycan (D1-S1) is liberated from the cartilage or better retained in the joint cavity. As discussed above the hydrops of joints operated on, may result in an underestima-

tion of the differences between the two joints. With some animals the concentration of proteoglycan antigens in the left and right joint appeared rather similar perhaps as an effect of the hydrops. It is also possible that lameness of some of the animals change the load of their right joint such that degenerative changes are induced with an increased fragmentation and liberation of proteoglycans.

Comparison of concentrations of proteoglycan antigens in the synovial fluids at different sampling times with the preoperative sample of the same joint showed higher concentrations of proteoglycans in the synovial fluid after operation, Table I and II, Fig. 2. Again, however, the differences were less pronounced with the keratan sulphate rich proteoglycans (D1-S2) (not shown in the Figure) compared with the chondroitin sulphate rich proteoglycans (D1-S1).

General discussion

Quantitation of proteoglycan fragments released to the synovial fluids in degenerative joint disease showed increased concentrations in diseased joints. Therefore, the procedure described can probably become useful for monitoring progression of destruction of articular cartilage. In addition, such procedures can be used in studies of effects of various forms of therapy on progression of the disease. Problems that should be solved, before extensive use of the procedure, include measurement of the volume of the synovial fluid and preferably also rate of elimination of the fragments from the fluid. Separate experiments indicate that a similar ELISA procedure can be used for quantitation of cartilage proteoglycan fragments released from human articular cartilage. Two patients with yersinia arthritis and pyrophosphate arthritis had fragment concentrations 3 to 5 times higher compared with two patients where no involvement of joint structures could be shown.

The exact nature of the proteoglycan antigens released to the synovial fluid has not been further studied. They may therefore represent either intact or fragmented molecules. It has previously been shown that proteoglycans are degraded and that fragments of proteoglycans appear to be present in the articular cartilage during early stages of induced hip joint osteoarthritis (4). Consequently it is likely that the antigens in the synovial fluid that react in the assay represent fragmented proteoglycans.

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Table I. Concentration of proteoglycan fragments in synovial fluid samples. Coat with chondroitin sulfate rich proteoglycan (D1-S1) values expressed as $\mu\text{g/ml}$ of proteoglycan equivalents.

SYNOVIAL FLUID SAMPLING TIME (WEEK POSTOP)	DOG NUMBER																		DIFFERENCE BETWEEN THIS SAMPLE AND THE PREOP SAMPLE (DOGS I - IV)
	I		II		III		IV		V		VI		VII		VIII		IX		
LEFT	RIGHT	LEFT	RIGHT	LEFT	RIGHT	LEFT	RIGHT	LEFT	RIGHT	LEFT	RIGHT	LEFT	RIGHT	LEFT	RIGHT	LEFT	RIGHT	LEFT	RIGHT
PREOPERATIVE	77	--	37	--	71	--	72	--	--	--	--	--	--	--	--	--	--	--	--
3	252	107	83	81	--	--	177	--	105	26	73	--	--	--	90	--	23	--	P = 0.1
6	137	--	95	73	134	123	123	--	113	82	105	--	71	37	84	--	50	24	P < 0.025
10	152	73	87	--	89	104	67	--	--	--	76	32	65	19	--	--	48	35	P < 0.15
15	179	30	74	88	230	--	128	--	205	--	83	--	52	32	--	--	31	29	P < 0.05
19	135	90	94	111	149	--	198	--	70	--	94	--	69	61	118	73	39	--	P < 0.025
26	86	44	73	--	--	--	88	--	115	--	48	29	60	--	53	--	26	33	P < 0.15
29	--	291	145	--	63	41	121	--	75	23	28	19	79	--	--	--	35	--	P > 0.2

Table II. Concentration of proteoglycan fragments in synovial fluid samples. Coat with keratan sulfate rich proteoglycan (D1-S2) values expressed as $\mu\text{g/ml}$ of proteoglycan equivalents.

SYNOVIAL FLUID SAMPLING TIME (WEEK POSTOP)	DOG NUMBER															
	I		II		III		IV		V		VI		VII		VIII	
	LEFT	RIGHT	LEFT	RIGHT	LEFT	RIGHT	LEFT	RIGHT	LEFT	RIGHT	LEFT	RIGHT	LEFT	RIGHT	LEFT	RIGHT
PREOPERATIVE	49	--	53	--	55	--	56	--	--	--	--	--	--	--	--	--
3	137	41	74	84	--	--	168	--	104	40	29	--	--	--	60	43
5	54	--	134	49	86	70	148	--	88	103	58	--	79	43	119	68
10	66	58	81	--	68	61	53	--	--	--	45	31	58	32	--	37
15	61	27	69	101	127	--	89	--	166	--	53	--	58	43	--	53
19	53	--	94	81	75	--	94	--	95	--	52	--	57	92	94	38
26	42	34	45	--	--	--	80	--	93	--	25	24	50	--	42	30
29	--	95	117	--	40	38	92	--	103	38	27	27	114	--	--	37

DIFFERENCE BETWEEN
THIS SAMPLE AND THE
PREOP SAMPLE
(DOGS I-IV)

$P < 0.15$

$P < 0.1$

$P < 0.15$

$P < 0.1$

$P < 0.1$

$P > 0.2$

$P > 0.2$

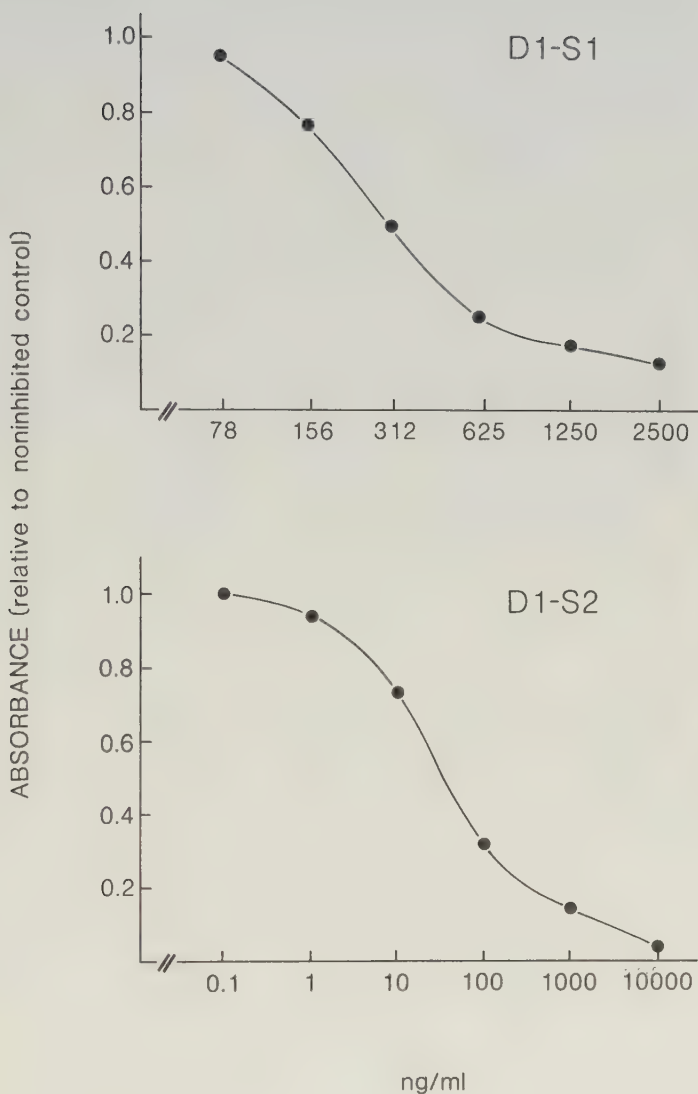


Fig. 1. Standard curves for ELISA of canine articular cartilage proteoglycans. Coat with bovine nasal cartilage D1-S1 (top) and D1-S2 (bottom). The antiserum was raised against canine articular cartilage proteoglycans, that were also used for inhibition after prior digestion with chondroitinase.

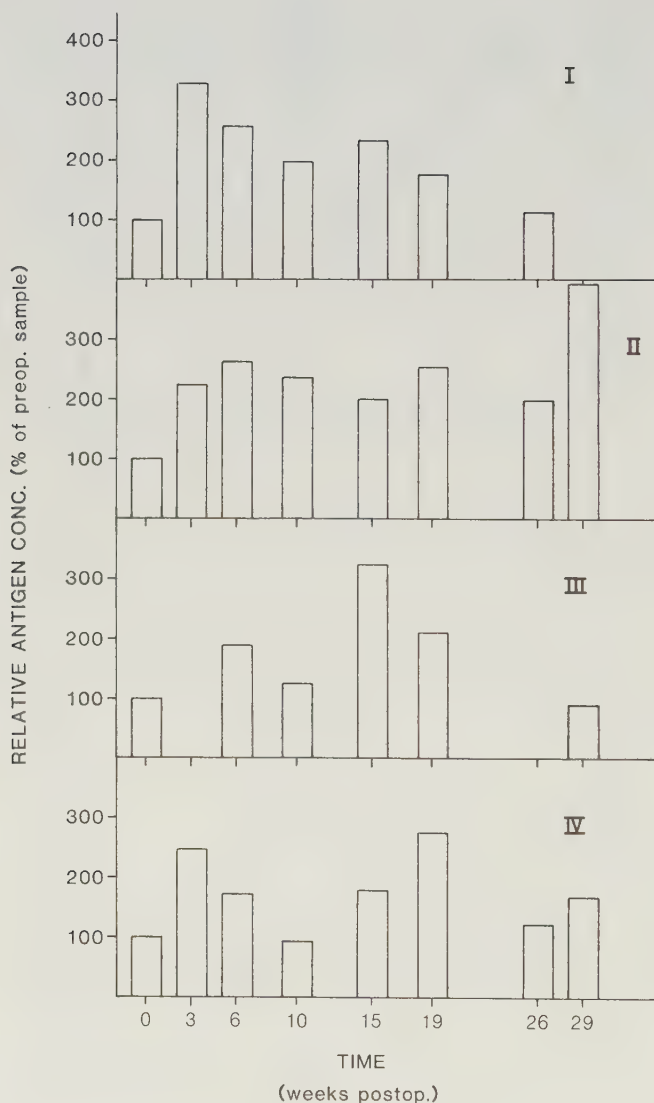


Fig. 2. Relative concentration of proteoglycan (fragments) in synovial fluid samples drawn at different sampling times. Concentrations expressed as ratio to the concentration in the preoperative sample. Coat with D1-S1, *i.e.* bovine chondroitin sulphate rich proteoglycans. No correction for dilution due to hydrops has been made.

